PATENT COOPERATION TREATY

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PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference PUR-48:PCT	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US97/07663	International filing date *(day/month/year)* 06 MAY 1997	Priority date *(day/month/year)* 06 MAY 1996
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant PURDUE RESEARCH FOUNDATION		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 9 sheets.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
05 DECEMBER 1997	30 JULY 1998
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer GABRIELE E. BUGAISKY
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (January 1994)★

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US97/07663

I. Basis of the report

1. This report has been drawn on the basis of *(Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments)*:

[X] the international application as originally filed.

[X] the description, pages 1-44 , as originally filed.
pages NONE , filed with the demand.
pages NONE , filed with the letter of _____.
pages _____ , filed with the letter of _____.

[X] the claims, Nos. 1-30 , as originally filed.
Nos. NONE , as amended under Article 19.
Nos. NONE , filed with the demand.
Nos. NONE , filed with the letter of _____.
Nos. _____ , filed with the letter of _____.

[X] the drawings, sheets/~~fig~~ 1-12 , as originally filed.
sheets/~~fig~~ NONE , filed with the demand.
sheets/~~fig~~ NONE , filed with the letter of _____.
sheets/~~fig~~ _____ , filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

[X] the description, pages NONE .
[X] the claims, Nos. NONE .
[X] the drawings, sheets/~~fig~~ NONE .

3. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the ~~Supplemental Box~~ Additional observations below (Rule 70.2(c)).

4. Additional observations, if necessary:
NONE

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US97/07663

V. Reasoned statement under Article 35(2) with regard t n velty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims	1-13, 17, 20-27, 29	YES
	Claims	14-16, 18-19, 28, 30	NO
Inventive Step (IS)	Claims	NONE	YES
	Claims	1-30	NO
Industrial Applicability (IA)	Claims	1-30	YES
	Claims	NONE	NO

2. CITATIONS AND EXPLANATIONS

Claims 14-16, 18-19, 28 and 30 lack novelty under PCT Article 33(2) as being anticipated by Yamano *et al.* The reference is anticipatory because it provides a plasmid containing an *Acetobacter aceti* ssp. *xylinum* α-actetolactate decarboxylase (ALDC)gene integrated into a ribosomal RNA gene of brewer's yeast (*Saccharomyces carlsbergensis*; ribosomal genes are known to integrate as multiple copies. The plasmid was successfully integrated into the genome of a strain of brewer's yeast; cells which expressed the exogenous gene could be maintained in non-selective medium for over 60 generations. The cells were co-transformed with a plasmid for g418 resistance (pZNEO). The proportion of ALDC positive clones was highest when the ratio of the ALDC integration cassette to pZNEO was 3:1. The response to the written opinion stated that the Examiner's conclusion was incorrect as instant invention includes not only transforming cells with a plasmid which is both replicative and integrative, but also repeatedly replicating the cells from the transformation to produce a number of generations of progeny while selecting for cells which include the selection marker; it was stated that none of the references teach, suggest or disclose such a process in which the cells are repeatedly replicated over a number of generations while maintaining the selection pressure. A careful review of the claimed invention leads the Examiner to conclude the initial opinion is correct. Cells in culture replicate through multiple generations; there is nothing in the claims which excludes cell division in culture; likewise, the claims do not explicitly state if or how the selection pressure is maintained throughout the culture process. The claims merely recite that there is some selection for cells which retain the marker; this can be construed as selecting those cells which retain the selection marker at the end of the culture period, as was the case for ALDC expression. From the comments to the written opinion, it appears that Applicants' intent was to view the claims as more narrowly drafted than they in fact seem to be written. If the language of the claims reflected the apparent intent, then the above reference would not be considered anticipatory. Rather the above reference, in combination with Rine *et al.*, who showed gene dosage effects through continued (Continued on Supplemental Sheet.)

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

CLASSIFICATION:
The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(6): C12N 1/16, 1/18, 1/19, 15/09; 15/68, 15/69, 15/81; C12P 7/06 and US Cl.: 435/161, 172.3, 254.2, 254.21, 320.1

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
selection pressure, would render such a claimed invention obvious.

Claims 14-16, 18-19, and 28 lack novelty under PCT Article 33(2) as being anticipated by Le Dall *et al.* The reference is anticipatory because it provides a plasmid containing a *Ura3* gene, and the *XPR2* gene encoding alkaline extracellular proteinase integrated into a ribosomal RNA gene of *Y. lipolytica*. Multiple copies of the plasmid were successfully integrated into the genome; cells which expressed the *Ura3* gene could be maintained in non-selective medium for at least 20 generations.
The response to the written opinion stated that the Examiner's conclusion was incorrect as instant invention includes not only transforming cells with a plasmid which is both replicative and integrative, but also repeatedly replicating the cells from the transformation to produce a number of generations of progeny while selecting for cells which include the selection marker; it was stated that none of the references teach, suggest or disclose such a process in which the cells are repeatedly replicated over a number of generations while maintaining the selection pressure. It appears that the reference may not have been fully appreciated...see e.g., the statement on p 39, paragraph 2 of the reference: "Our aim was... multi-copy integration system for use in *Y. lipolytica*. These plasmids contain...as well as derivatives of the *Y. lipolytica*...In addition these plasmids contained the *XPR2* gene used as a model for gene expression and protein secretion."

Claims 14-16, 18-19, 28 and 30 lack novelty under PCT Article 33(2) as being anticipated by Fujii *et al.* The reference is anticipatory because it provides a plasmid containing an *Acetobacter aceti* ssp. *xylinum* α-actetolactate decarboxylase gene integrated into a ribosomal RNA gene of brewer's yeast. Multiple copies of the plasmid were successfully integrated into the genome of a strain of brewer's yeast; cells which expressed the exogenous gene at low levels and had excised the marker sequences could be maintained in non-selective medium for over 80 generations. The response to the written opinion stated that the Examiner's conclusion was incorrect as instant invention includes not only transforming cells with a plasmid which is both replicative and integrative, but also repeatedly replicating the cells from the transformation to produce a number of generations of progeny while selecting for cells which include the selection marker; it was stated that none of the references teach, suggest or disclose such a process in which the cells are repeatedly replicated over a number of generations while maintaining the selection pressure. Please see figure 1 of the reference which shows the plasmid pIARL28. It contains the ALDC gene, with sequences for ribosomal insertion and also the G418 resistance gene for selection. On p 998,column 2 it is stated "Integrants were selected on the basis of uracil prototrophy or resistance to G418 respectively. The number of transformants obtained...This result indicated that the rDNA genes were useful target sequences because they enhanced integration efficiency due to their high copy number in the genome."

Claims 14-16, 18-19, 28 and 30 lack novelty under PCT Article 33(2) as being anticipated by Lopes *et al.* The reference is anticipatory because it provides numerous plasmid containing various genes integrated into a ribosomal RNA gene of *Saccharomyces cerevisiae*. Multiple copies of the plasmid were successfully integrated into the genome; cells were maintained in non-selective medium for multiple generations and stability of the integrated genes was assessed. Some of the factors affecting mitotic stability were addressed. The response to the written opinion stated that the Examiner's conclusion was incorrect as instant invention includes not only transforming cells with a plasmid which is both replicative and integrative, but also repeatedly replicating the cells from the transformation to produce a number of generations of progeny while selecting for cells which include the selection marker; it was stated that none of the references teach, suggest or disclose such a process in which the cells are repeatedly replicated over a number of generations while maintaining the selection pressure. It is noted that Figure 1 shows the plasmids contain not only the *Leu2d* selection marker, but also various cloned genes for stability and expression studies. on p 468, paragraph 1, it is stated Yeast transformants were selected by plating on...The same medium was used for growing the transformants in liquid culture". It would appear the reference is indeed anticipatory.

Claims 1-29 lack an inventive step under PCT Article 33(3) as being obvious over any of Yamano *et al.*, Le Dall *et al.*, Fujii *et al.*, or Lopes *et al.* in view of Tantirungkij *et al.* (Appl. Micro. Biotech.). The teachings of Yamano *et al.*, Le Dall *et al.*, Fujii *et al.* or Lopes *et al.* are each discussed above; none describes a yeast containing the genes for xylose fermentation multiply integrated into the ribosomal genes. Tantirungkij *et al.* describe recombinant *Saccharomyces cerevisiae* which contain and express the genes for xylose assimilation integrated into the genome, albeit not into the ribosomal genes. In order to obtain a higher copy number of the genes for xylose assimilation and thus higher expression levels than observed by Tantirungkij *et al.*, it would have been obvious for one of ordinary skill in the art at the time of the invention to place the

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

xylose assimilation genes into a ribosomal integration vector, as taught by any of Yamano *et al.*, Le Dall *et al.*, Fujii *et al.*, or Lopes *et al.*, with a reasonable expectation of success in the venture. It was stated in the response to the written opinion that, as mentioned above, the references cited in the novelty rejections fail to teach critical aspects of the claimed invention, and that none of the addition references teaches or discloses the processes as claimed. As discussed above, the Examiner disagrees with the Applicants' conclusions for those references cited as anticipatory, and that the combination of references renders the instant claims obvious.

Claims 1-2 and 4-29 lack an inventive step under PCT Article 33(3) as being obvious over Le Dall *et al.* in view of Tantirungkij *et al.* (Appl. Micro. Biotech.). The teachings of Le Dall *et al.* are discussed above; they do not describe a *Yarrowia* yeast containing the genes for xylose fermentation multiply integrated into the ribosomal genes. Tantirungkij *et al.* describe recombinant *Saccharomyces cerevisiae* which contain and express the genes for xylose assimilation integrated into the genome, albeit not into the ribosomal genes. In order to *Yarrowia lipolytica* cells which demonstrate high levels of xylose assimilation, it would have been obvious for one of ordinary skill in the art at the time of the invention to place the xylose assimilation genes of Tantirungkij *et al.* into the ribosomal integration vector of Le Dall *et al.*, with a reasonable expectation of success in the venture. It was stated in the response to the written opinion that, as mentioned above, the references cited in the novelty rejections fail to teach critical aspects of the claimed invention, and that none of the addition references teaches or discloses the processes as claimed. As discussed above, the Examiner disagrees with the Applicants' conclusions for those references cited as anticipatory, and that the combination of references renders the instant claims obvious.

Claims 1-29 lack an inventive step under PCT Article 33(3) as being obvious over any of Yamano *et al.*, Le Dall *et al.*, Fujii *et al.*, or Lopes *et al.* in view of Tantirungkij *et al.* (Ann. N.Y. Acad. Sci.). The teachings of Yamano *et al.*, Le Dall *et al.*, Fujii *et al.* or Lopes *et al.* are each discussed above; none describes a yeast containing the genes for xylose fermentation multiply integrated into the ribosomal genes. Tantirungkij *et al.* describe recombinant *Saccharomyces cerevisiae* which contain and express the genes for xylose assimilation integrated into the genome, albeit not into the ribosomal genes. In order to obtain a higher copy number of the genes for xylose assimilation and thus higher expression levels than observed by Tantirungkij *et al.*, it would have been obvious for one of ordinary skill in the art at the time of the invention to place the xylose assimilation genes into a ribosomal integration vector, as taught by any of Yamano *et al.*, Le Dall *et al.*, Fujii *et al.*, or Lopes *et al.*, with a reasonable expectation of success in the venture. It was stated in the response to the written opinion that, as mentioned above, the references cited in the novelty rejections fail to teach critical aspects of the claimed invention, and that none of the addition references teaches or discloses the processes as claimed. As discussed above, the Examiner disagrees with the Applicants' conclusions for those references cited as anticipatory, and that the combination of references renders the instant claims obvious.

Claims 1-2 and 4-29 lack an inventive step under PCT Article 33(3) as being obvious over Le Dall *et al.* in view of Tantirungkij *et al.* (Ann. N.Y. Acad. Sci.). The teachings of Le Dall *et al.* are discussed above; they do not describe a *Yarrowia* yeast containing the genes for xylose fermentation multiply integrated into the ribosomal genes. Tantirungkij *et al.* describe recombinant *Saccharomyces cerevisiae* which contain and express the genes for xylose assimilation integrated into the genome, albeit not into the ribosomal genes. In order to *Yarrowia lipolytica* cells which demonstrate high levels of xylose assimilation, it would have been obvious for one of ordinary skill in the art at the time of the invention to place the xylose assimilation genes of Tantirungkij *et al.* into the ribosomal integration vector of Le Dall *et al.*, with a reasonable expectation of success in the venture. It was stated in the response to the written opinion that, as mentioned above, the references cited in the novelty rejections fail to teach critical aspects of the claimed invention, and that none of the addition references teaches or discloses the processes as claimed. As discussed above, the Examiner disagrees with the Applicants' conclusions for those references cited as anticipatory, and that the combination of references renders the instant claims obvious.

Claims 1-29 lack an inventive step under PCT Article 33(3) as being obvious over any of Yamano *et al.*, Le Dall *et al.*, Fujii *et al.*, or Lopes *et al.* in view of WO 95/13362 (Purdue Research Foundation). The teachings of Yamano *et al.*, Le Dall *et al.*, Fujii *et al.* or Lopes *et al.* are each discussed above; none describes a yeast containing the genes for xylose fermentation multiply integrated into the ribosomal genes. WO 95/13362 describes recombinant *Saccharomyces cerevisiae* which contain and express the genes for xylose assimilation integrated into the genome, albeit not into the ribosomal genes. In order to obtain a higher copy number of the genes for xylose assimilation and thus higher expression levels than observed by WO 95/13362, it would have been obvious for one of ordinary skill in the art at the time of the invention to place the xylose assimilation genes into a ribosomal integration vector, as taught by any of Yamano *et al.*, Le Dall *et al.*, Fujii *et al.*, or

Supplemental B x
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Lopes *et al.*, with a reasonable expectation of success in the venture. It was stated in the response to the written opinion that, as mentioned above, the references cited in the novelty rejections fail to teach critical aspects of the claimed invention, and that none of the addition references teaches or discloses the processes as claimed. As discussed above, the Examiner disagrees with the Applicants' conclusions for those references cited as anticipatory, and that the combination of references renders the instant claims obvious.

Claims 1-2 and 4-29 lack an inventive step under PCT Article 33(3) as being obvious over Le Dall *et al.* in view of WO 95/13362 (Purdue Research Foundation). The teachings of Le Dall *et al.* are discussed above; they do not describe a *Yarrowia* yeast containing the genes for xylose fermentation multiply integrated into the ribosomal genes. WO 95/13362 describes recombinant *Saccharomyces cerevisiae* which contain and express the genes for xylose assimilation integrated into the genome, albeit not into the ribosomal genes. In order to *Yarrowia lipolytica* cells which demonstrate high levels of xylose assimilation, it would have been obvious for one of ordinary skill in the art at the time of the invention to place the xylose assimilation genes of WO 95/13362 into the ribosomal integration vector of Le Dall *et al.*, with a reasonable expectation of success in the venture. It was stated in the response to the written opinion that, as mentioned above, the references cited in the novelty rejections fail to teach critical aspects of the claimed invention, and that none of the addition references teaches or discloses the processes as claimed. As discussed above, the Examiner disagrees with the Applicants' conclusions for those references cited as anticipatory, and that the combination of references renders the instant claims obvious.

Claims 1-30 meet the criteria set out in PCT Article 33(4), because it would be useful to have yeasts that efficiently use both xylose and glucose for growth.

NEW CITATIONS

Rine et al. Targeted selection of recombinant clones through gene dosage effects. Proceedings of the National Academy of Sciences, USA, November 1983, Vol. 80, pages 6750-6754, entire document.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference PUR-48:PCT	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US97/07663	International filing date *(day/month/year)* 06 MAY 1997	(Earliest) Priority Date *(day/month/year)* 06 MAY 1996
Applicant PURDUE RESEARCH FOUNDATION		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (See Box I).

2. [ ] **Unity of invention is lacking** (See Box II).

3. [ ] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [ ] filed with the international application.
   - [ ] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] transcribed by this Authority.

4. With regard to the title,
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established by this Authority to read as follows:

5. With regard to the abstract,
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is:

   Figure No. _____
   - [ ] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.
   - [X] None of the figures.

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under Article 19. The Notes are based on the requirements of the Patent Cooperation Treaty and of the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule" and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended ?

The claims only.

The description and the drawings may only be amended during international preliminary examination under Chapter II.

When ? Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments ?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How ? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

What documents must/may accompany the amendments ?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confounded with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

(i) the claim is unchanged;
(ii) the claim is cancelled;
(iii) the claim is new;
(iv) the claim replaces one or more claims as filed;
(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under Article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

The statement should be brief, it should not exceed 500 words if in English or if translated into English.

It should not be confounded with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It should not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

In what language ?

The amendments must be made in the language in which the international application is published. The letter and any statement accompanying the amendments must be in the same language as the international application if that language is English or French; otherwise, it must be in English or French, at the choice of the applicant.

Consequence if a demand for international preliminary examination has already been filed ?

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase ?

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 12 February 1998 (12.02.98)	
International application No. PCT/US97/07663	**Applicant's or agent's file reference** PUR-48:PCT
International filing date (day/month/year) 06 May 1997 (06.05.97)	**Priority date** (day/month/year) 06 May 1996 (06.05.96)
Applicant HO, Nancy, W., Y. et al	

1. The designated Office is hereby notified of its election made:

   [X] in the demand filed with the International Preliminary Examining Authority on:

   05 December 1997 (05.12.97)

   [ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

   [ ] was not

   made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer A. Addae-Ruesch
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: KENNETH A. GANDY
WOODARD, EMHARDT, NAUGHTON, MORIARTY & MCNETT
BANK ONE CENTER/TOWER, SUITE 3700
111 MONUMENT CIRCLE
INDIANAPOLIS, IN 46204

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing *(day/month/year)*	02 SEP 1998
Applicant's or agent's file reference PUR-48:PCT	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority Date *(day/month/year)*
PCT/US97/07663	06 MAY 1997	06 MAY 1996

Applicant
PURDUE RESEARCH FOUNDATION

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	GABRIELE E. BUGAISKY
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/416 (July 1992)★

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference PUR-48:PCT	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US97/07663	International filing date *(day/month/year)* 06 MAY 1997	Priority date *(day/month/year)* 06 MAY 1996
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant PURDUE RESEARCH FOUNDATION		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 6 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report

   II ☐ Priority

   III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV ☐ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☐ Certain defects in the international application

   VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
05 DECEMBER 1997	30 JULY 1998
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer GABRIELE E. BUGAISKY Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (January 1994)*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US97/07663

I. Basis of the report

1. This report has been drawn on the basis of (*Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments*):

[X] the international application as originally filed.

[X] the description, pages 1-44 , as originally filed.
pages NONE , filed with the demand.
pages NONE , filed with the letter of _____.
pages _____ , filed with the letter of _____.

[X] the claims, Nos. 1-30 , as originally filed.
Nos. NONE , as amended under Article 19.
Nos. NONE , filed with the demand.
Nos. NONE , filed with the letter of _____.
Nos. _____ , filed with the letter of _____.

[X] the drawings, sheets/~~fig~~ 1-12 , as originally filed.
sheets/~~fig~~ NONE , filed with the demand.
sheets/~~fig~~ NONE , filed with the letter of _____.
sheets/~~fig~~ _____ , filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

[X] the description, pages NONE .
[X] the claims, Nos. NONE .
[X] the drawings, sheets/~~fig~~ NONE .

3. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the ~~Supplemental Box~~ Additional observations below (Rule 70.2(c)).

4. Additional observations, if necessary:

NONE

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US97/07663

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims	1-13, 17, 20-27, 29	YES
	Claims	14-16, 18-19, 28, 30	NO
Inventive Step (IS)	Claims	NONE	YES
	Claims	1-30	NO
Industrial Applicability (IA)	Claims	1-30	YES
	Claims	NONE	NO

2. CITATIONS AND EXPLANATIONS

Claims 14-16, 18-19, 28 and 30 lack novelty under PCT Article 33(2) as being anticipated by Yamano *et al.* The reference is anticipatory because it provides a plasmid containing an *Acetobacter aceti* ssp. *xylinum* α-actetolactate decarboxylase (ALDC)gene integrated into a ribosomal RNA gene of brewer's yeast (*Saccharomyces carlsbergensis*; ribosomal genes are known to integrate as multiple copies. The plasmid was successfully integrated into the genome of a strain of brewer's yeast; cells which expressed the exogenous gene could be maintained in non-selective medium for over 60 generations. The cells were co-transformed with a plasmid for g418 resistance (pZNEO). The proportion of ALDC positive clones was highest when the ratio of the ALDC integration cassette to pZNEO was 3:1. The response to the written opinion stated that the Examiner's conclusion was incorrect as instant invention includes not only transforming cells with a plasmid which is both replicative and integrative, but also repeatedly replicating the cells from the transformation to produce a number of generations of progeny while selecting for cells which include the selection marker; it was stated that none of the references teach, suggest or disclose such a process in which the cells are repeatedly replicated over a number of generations while maintaining the selection pressure. A careful review of the claimed invention leads the Examiner to conclude the initial opinion is correct. Cells in culture replicate through multiple generations; there is nothing in the claims which excludes cell division in culture; likewise, the claims do not explicitly state if or how the selection pressure is maintained throughout the culture process. The claims merely recite that there is some selection for cells which retain the marker; this can be construed as selecting those cells which retain the selection marker at the end of the culture period, as was the case for ALDC expression. From the comments to the written opinion, it appears that Applicants' intent was to view the claims as more narrowly drafted than they in fact seem to be written. If the language of the claims reflected the apparent intent, then the above reference would not be considered anticipatory. Rather the above reference, in combination with Rine *et al.*, who showed gene dosage effects through continued (Continued on Supplemental Sheet.)

Supplemental B x
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

CLASSIFICATION:
The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(6): C12N 1/16, 1/18, 1/19, 15/09; 15/68, 15/69, 15/81; C12P 7/06 and US Cl.: 435/161, 172.3, 254.2, 254.21, 320.1

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
selection pressure, would render such a claimed invention obvious.

Claims 14-16, 18-19, and 28 lack novelty under PCT Article 33(2) as being anticipated by Le Dall *et al.* The reference is anticipatory because it provides a plasmid containing a *Ura3* gene, and the *XPR2* gene encoding alkaline extracellular proteinase integrated into a ribosomal RNA gene of *Y. lipolytica*. Multiple copies of the plasmid were successfully integrated into the genome; cells which expressed the *Ura3* gene could be maintained in non-selective medium for at least 20 generations.
The response to the written opinion stated that the Examiner's conclusion was incorrect as instant invention includes not only transforming cells with a plasmid which is both replicative and integrative, but also repeatedly replicating the cells from the transformation to produce a number of generations of progeny while selecting for cells which include the selection marker; it was stated that none of the references teach, suggest or disclose such a process in which the cells are repeatedly replicated over a number of generations while maintaining the selection pressure. It appears that the reference may not have been fully appreciated...see e.g., the statement on p 39, paragraph 2 of the reference: "Our aim was... multi-copy integration system for use in *Y. lipolytica*. These plasmids contain...as well as derivatives of the *Y. lipolytica*...In addition these plasmids contained the *XPR2* gene used as a model for gene expression and protein secretion."

Claims 14-16, 18-19, 28 and 30 lack novelty under PCT Article 33(2) as being anticipated by Fujii *et al.* The reference is anticipatory because it provides a plasmid containing an *Acetobacter aceti* ssp. *xylinum* α-actetolactate decarboxylase gene integrated into a ribosomal RNA gene of brewer's yeast. Multiple copies of the plasmid were successfully integrated into the genome of a strain of brewer's yeast; cells which expressed the exogenous gene at low levels and had excised the marker sequences could be maintained in non-selective medium for over 80 generations. The response to the written opinion stated that the Examiner's conclusion was incorrect as instant invention includes not only transforming cells with a plasmid which is both replicative and integrative, but also repeatedly replicating the cells from the transformation to produce a number of generations of progeny while selecting for cells which include the selection marker; it was stated that none of the references teach, suggest or disclose such a process in which the cells are repeatedly replicated over a number of generations while maintaining the selection pressure. Please see figure 1 of the reference which shows the plasmid pIARL28. It contains the ALDC gene, with sequences for ribosomal insertion and also the G418 resistance gene for selection. On p 998,column 2 it is stated "Integrants were selected on the basis of uracil prototrophy or resistance to G418 respectively. The number of transformants obtained...This result indicated that the rDNA genes were useful target sequences because they enhanced integration efficiency due to their high copy number in the genome."

Claims 14-16, 18-19, 28 and 30 lack novelty under PCT Article 33(2) as being anticipated by Lopes *et al.* The reference is anticipatory because it provides numerous plasmid containing various genes integrated into a ribosomal RNA gene of *Saccharomyces cerevisiae*. Multiple copies of the plasmid were successfully integrated into the genome; cells were maintained in non-selective medium for multiple generations and stability of the integrated genes was assessed. Some of the factors affecting mitotic stability were addressed. The response to the written opinion stated that the Examiner's conclusion was incorrect as instant invention includes not only transforming cells with a plasmid which is both replicative and integrative, but also repeatedly replicating the cells from the transformation to produce a number of generations of progeny while selecting for cells which include the selection marker; it was stated that none of the references teach, suggest or disclose such a process in which the cells are repeatedly replicated over a number of generations while maintaining the selection pressure. It is noted that Figure 1 shows the plasmids contain not only the *Leu2d* selection marker, but also various cloned genes for stability and expression studies. on p 468, paragraph 1, it is stated Yeast transformants were selected by plating on...The same medium was used for growing the transformants in liquid culture". It would appear the reference is indeed anticipatory.

Claims 1-29 lack an inventive step under PCT Article 33(3) as being obvious over any of Yamano *et al.*, Le Dall *et al.*, Fujii *et al.*, or Lopes *et al.* in view of Tantirungkij *et al.* (Appl. Micro. Biotech.). The teachings of Yamano *et al.*, Le Dall *et al.*, Fujii *et al.* r Lopes *et al.* are each discussed above; none describes a yeast containing the genes f r xylose fermentation multiply integrated into the ribosomal genes. Tantirungkij *et al.* describe recombinant *Saccharomyces cerevisiae* which contain and express the genes for xylose assimilation integrated into the genome, albeit not into the ribosomal genes. In order to obtain a higher copy number f the genes for xylose assimilation and thus higher expression levels than observed by Tantirungkij *et al.*, it would have been obvious for one f ordinary skill in the art at the time of the invention to place th

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: B xes I - VIII

xylose assimilation genes into a ribosomal integration vector, as taught by any of Yamano *et al.*, Le Dall *et al.*, Pujii *et al.*, or Lopes *et al.*, with a reasonable expectation of success in the venture. It was stated in the response to the written opinion that, as mentioned above, the references cited in the novelty rejections fail to teach critical aspects of the claimed invention, and that none of the addition references teaches or discloses the processes as claimed. As discussed above, the Examiner disagrees with the Applicants' conclusions for those references cited as anticipatory, and that the combination of references renders the instant claims obvious.

Claims 1-2 and 4-29 lack an inventive step under PCT Article 33(3) as being obvious over Le Dall *et al.* in view of Tantirungkij *et al.* (Appl. Micro. Biotech.). The teachings of Le Dall *et al.* are discussed above; they do not describe a *Yarrowia* yeast containing the genes for xylose fermentation multiply integrated into the ribosomal genes. Tantirungkij *et al.* describe recombinant *Saccharomyces cerevisiae* which contain and express the genes for xylose assimilation integrated into the genome, albeit not into the ribosomal genes. In order to *Yarrowia lipolytica* cells which demonstrate high levels of xylose assimilation, it would have been obvious for one of ordinary skill in the art at the time of the invention to place the xylose assimilation genes of Tantirungkij *et al.* into the ribosomal integration vector of Le Dall *et al.*, with a reasonable xpectation of success in the venture. It was stated in the response to the written opinion that, as mentioned above, the references cited in the novelty rejections fail to teach critical aspects of the claimed invention, and that none of the addition references teaches or discloses the processes as claimed. As discussed above, the Examiner disagrees with the Applicants' conclusions for those references cited as anticipatory, and that the combination of references renders the instant claims obvious.

Claims 1-29 lack an inventive step under PCT Article 33(3) as being obvious over any of Yamano *et al.*, Le Dall *et al.*, Pujii *et al.*, or Lopes *et al.* in view of Tantirungkij *et al.* (Ann. N.Y. Acad. Sci.). The teachings of Yamano *et al.*, Le Dall *et al.*, Pujii *et al.* or Lopes *et al.* are each discussed above; none describes a yeast containing the genes for xylose fermentation multiply integrated into the ribosomal genes. Tantirungkij *et al.* describe recombinant *Saccharomyces cerevisiae* which contain and express the genes for xylose assimilation integrated into the genome, albeit not into the ribosomal genes. In order to obtain a higher copy number of the genes for xylose assimilation and thus higher expression levels than observed by Tantirungkij *et al.*, it would have been obvious for one of ordinary skill in the art at the time of the invention to place the xylose assimilation genes into a ribosomal integration vector, as taught by any of Yamano *et al.*, Le Dall *et al.*, Pujii *et al.*, or Lopes *et al.*, with a reasonable expectation of success in the venture. It was stated in the response to the written opinion that, as mentioned above, the references cited in the novelty rejections fail to teach critical aspects of the claimed invention, and that none of the addition references teaches or discloses the processes as claimed. As discussed above, the Examiner disagrees with the Applicants' conclusions for those references cited as anticipatory, and that the combination of references renders the instant claims obvious.

Claims 1-2 and 4-29 lack an inventive step under PCT Article 33(3) as being obvious over Le Dall *et al.* in view of Tantirungkij *et al.* (Ann. N.Y. Acad. Sci.). The teachings of Le Dall *et al.* are discussed above; they do not describe a *Yarrowia* yeast containing the genes for xylose fermentation multiply integrated into the ribosomal genes. Tantirungkij *et al.* describe recombinant *Saccharomyces cerevisiae* which contain and express the genes for xylose assimilation integrated into the genome, albeit not into the ribosomal genes. In order to *Yarrowia lipolytica* cells which demonstrate high levels of xylose assimilation, it would have been obvious for one of ordinary skill in the art at the time of the invention to place the xylose assimilation genes of Tantirungkij *et al.* into the ribosomal integration vector of Le Dall *et al.*, with a reasonable expectation of success in the venture. It was stated in the response to the written opinion that, as mentioned above, the references cited in the novelty rejections fail to teach critical aspects of the claimed invention, and that none of the addition references teaches or discloses the processes as claimed. As discussed above, the Examiner disagrees with the Applicants' conclusions for those references cited as anticipatory, and that the combination of references renders the instant claims obvious.

Claims 1-29 lack an inventive step under PCT Article 33(3) as being obvious over any of Yamano *et al.*, Le Dall *et al.*, Pujii *et al.*, or Lopes *et al.* in view of WO 95/13362 (Purdue Research Foundation). The teachings of Yamano *et al.*, Le Dall *et al.*, Pujii *et al.* or Lopes *et al.* are each discussed above; none describes a yeast containing the genes for xylose fermentation multiply integrated into the ribosomal genes. WO 95/13362 describes recombinant *Saccharomyces cerevisiae* which contain and express the genes for xylose assimilation integrated into the genome, albeit not into the ribosomal genes. In order to obtain a higher copy number f the genes for xylose assimilation and thus higher expression levels than observed by WO 95/13362, it would hav been obvious for one of ordinary skill in the art at the time of the invention to place the xylose assimilation genes into a ribosomal integration vector, as taught by any f Yamano *et al.*, Le Dall *et al.*, Pujii *et al.*, or

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Lopes *et al.*, with a reasonable expectation of success in the venture. It was stated in the response to the written opinion that, as mentioned above, the references cited in the novelty rejections fail to teach critical aspects of the claimed invention, and that none of the addition references teaches or discloses the processes as claimed. As discussed above, the Examiner disagrees with the Applicants' conclusions for those references cited as anticipatory, and that the combination of references renders the instant claims obvious.

Claims 1-2 and 4-29 lack an inventive step under PCT Article 33(3) as being obvious over Le Dall *et al.* in view of WO 95/13362 (Purdue Research Foundation). The teachings of Le Dall *et al.* are discussed above; they do not describe a *Yarrowia* yeast containing the genes for xylose fermentation multiply integrated into the ribosomal genes. WO 95/13362 describes recombinant *Saccharomyces cerevisiae* which contain and express the genes for xylose assimilation integrated into the genome, albeit not into the ribosomal genes. In order to *Yarrowia lipolytica* cells which demonstrate high levels of xylose assimilation, it would have been obvious for one of ordinary skill in the art at the time of the invention to place the xylose assimilation genes of WO 95/13362 into the ribosomal integration vector of Le Dall *et al.*, with a reasonable expectation of success in the venture. It was stated in the response to the written opinion that, as mentioned above, the references cited in the novelty rejections fail to teach critical aspects of the claimed invention, and that none of the addition references teaches or discloses the processes as claimed. As discussed above, the Examiner disagrees with the Applicants' conclusions for those references cited as anticipatory, and that the combination of references renders the instant claims obvious.

Claims 1-30 meet the criteria set out in PCT Article 33(4), because it would be useful to have yeasts that efficiently use both xylose and glucose for growth.

NEW CITATIONS

Rine et al. Targeted selection of recombinant clones through gene dosage effects. Proceedings of the National Academy of Sciences, USA, November 1983, Vol. 80, pages 6750-6754, entire document.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/07663

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 1/16, 1/18, 1/19, 15/09; 15/68, 15/69, 15/81; C12P 7/06
US CL :435/161, 172.3, 254.2, 254.21, 320.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/161, 172.3, 254.2, 254.21, 320.1; 536/23.2, 935/28, 37, 61

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	YAMANO, S. et al. Construction of a brewer's yeast having alpha-acetolactate decarboxylase gene of Acetobacter aceti ssp. xylinum integrated in the genome. J. Biotechnol. 14 February 1994. Vol. 32. No. 2. pages 173-178, entire document.	14-16, 18-19, 28, 30 ---------- 1-13, 17, 20-27, 29
X --- Y	LE DALL M.T. et al. Multiple copy integration in the yeast Yarrowia lipolytica. Curr. Genet. 1994. Vol. 26, pages 38-44, entire document.	14-16, 18-19, 28 ---------- 1-2, 5-6, 8-13. 17, 20-27, 29

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 19 JUNE 1997	Date of mailing of the international search report 28 JUL 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer GABRIELE B. BUGAISKY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/07663

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	FUJII et al. Application of a Ribosomal DNA Integration Vector in the Construction of a Brewer's Yeast Having α-Acetolactate Decarboxylase Activity. Applied and Environmental Microbiology. April, 1990. Vol. 56. No. 4, pages 997-1003, entire document.	14-16, 18- 19, 28, 30 --- 1-13, 17, 20-27, 29
Y	TANTIRUNGKIJ et al. Fed-batch fermentation of xylose by a fast-growing mutant of xylose-assimilating recombinant Saccharomyces cerevisiae. Applied Microbiology and Biotechnology. 1994. Vol. 41. No.1. pages 8-12, entire document.	1-29
Y	TANTIRUNGKIJ et al. Genetic Improvement of Saccharomyces cerevisiae for Ethanol Production from Xylose. Ann. N. Y. Acad. Sci. 02 May 1994, Vol. 721, pages 138-147, entire document.	1-29
Y	WO 95/13362 A1 (PURDUE RESEARCH FOUNDATION) 18 May 1995. entire document.	1-29
X --- Y	LOPES et al. Factors affecting the mitotic stability of high copy number integration into the ribosomal DNA of Saccharomyces cerevisiae. Yeast. 1996. Vol. 12. pages 467-477, entire document.	14-16, 18-19, 28, 30 --- 1-13, 17, 20-27, 29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/07663

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, Dialog files 301, 155, 5, 351 (Chemname, Medline, Biosis, Derwent WPI)
search terms: Xyulokinase, xylose reductase, xylitol dehydrogenase, vector?, plasmid?, (ribosom? or 18Sor 28S(3n)(gene? ? or chromosom? or locus or loci or cistron), integrat?, insert?, yeast, Saccharomyces, Candida, Pichia, Pachysolen, ferment? degrad?, xylose, nonselect?

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* PUR-48:PCT

Box No. I TITLE OF INVENTION

STABLE RECOMBINANT YEASTS FOR FERMENTING XYLOSE TO ETHANOL

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

PURDUE RESEARCH FOUNDATION
Office of Technology Transfer
1063 Hovde Hall
West Lafayette, Indiana 47907
United States of America

☐ This person is also inventor.

Telephone No. 317-494-2610

Facsimile No.

Teleprinter No.

State *(i.e. country)* of nationality: US

State *(i.e. country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

HO, Nancy W.Y.
606 Riley Lane
West Lafayette, Indiana 47906
United States of America

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: US

State *(i.e. country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

GANDY, Kenneth A.
WOODARD, EMHARDT, NAUGHTON, MORIARTY & MCNETT
Bank One Center/Tower, Suite 3700
111 Monument Circle
Indianapolis, Indiana 46204 US

SEE CONTINUATION TO BOX NO. IV ON SHEET NO. 4

Telephone No. 317-634-3456

Facsimile No. 317-637-7561

Teleprinter No. 810-341-3283

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

STABLE RECOMBINANT YEASTS FOR FERMENTING XYLOSE TO ETHANOL

BACKGROUND

The present invention relates generally to genetically engineered microorganisms and in particular to unique methods for stably incorporating exogenous DNA into cells, including the incorporation of multiple copies of the exogenous DNA at reiterated DNA sequences in the host. In a preferred aspect, the invention relates to yeasts capable of fermenting xylose (preferably cofermenting the same with glucose) to ethanol. More particularly, a preferred aspect of the invention relates to yeasts containing cloned genes encoding xylose reductase (XR), xylitol dehydrogenase (XD), and xylulokinase (XK), which yeasts substantially retain their efficiency for fermenting xylose to ethanol even after culturing in non-selective medium for a large number of generations.

As further background, recent studies have proven ethanol to be an ideal liquid fuel for automobiles. It can be used directly as a neat fuel (100% ethanol) or as a blend with gasoline at various concentrations. The use of ethanol to supplement or replace gasoline can reduce the dependency of many nations on imported foreign oil and also provide a renewable fuel for transportation. Furthermore, ethanol has proven to provide cleaner fuels that release far fewer pollutants into the environment than regular gasoline. For example, it has been demonstrated that the use of oxygenated materials in gasoline can reduce the emission of carbon monoxide, a

harmful pollutant, into the air. Among the several oxygenates currently used for boosting the oxygen content of gasoline, ethanol has the highest oxygen content. The United States Environmental Protection Agency (EPA) has shown that gasoline blended with 10% ethanol reduces carbon monoxide emissions by about 25% - 30%.

Up to now, the feedstock used for the production of industrial alcohol by fermentation has been sugars from sugar cane or beets and starch from corn or other food crops. However, these agricultural crops are presently considered to be too expensive to be used as feedstock for the large-scale production of fuel ethanol. Plant biomass is an attractive feedstock for ethanol-fuel production by fermentation because it is renewable, and available at low costs and in large amounts. The concept of using alcohol produced by microbial fermentation of sugars from agricultural biomass had its nascence at least two decades ago. The major fermentable sugars from cellulosic materials are glucose and xylose, with the ratio of glucose to xylose being approximately 2 or 3 to 1. The most desirable fermentations of cellulosic materials would, of course, completely convert both glucose and xylose to ethanol. Unfortunately, even now there is not a single known natural microorganism capable of fermenting both glucose and xylose effectively.

Yeasts, particularly *Saccharomyces* yeasts, have traditionally been used for fermenting glucose-based feedstocks to ethanol, and they are still considered the best microorganisms for that purpose. However, these glucose-fermenting yeasts, including the *Saccharomyces*

yeasts, have been found to be unable to ferment xylose and also unable to use this pentose sugar for growth.

Recently, N. Ho et al. have developed recombinant yeasts, particularly recombinant *Saccharomyces* yeasts, capable of effectively fermenting xylose to ethanol (Ho and Tsao, 1995). More particularly, the preferred recombinant yeasts were capable of co-fermenting the two major sugar constituents of cellulosic biomass, glucose and xylose, to ethanol (Ho and Tsao, 1995). These recombinant yeasts were developed by the transformation of yeasts with a high-copy number plasmid containing three cloned genes, XR, XD, and XK, encoding three key enzymes for xylose metabolism (Figure 1). Figure 2 and Figure 3 demonstrate two of the prior-made recombinant *Saccharomyces* yeasts, designated 1400 (pLNH32) and 1400(pLNH33), capable of co-fermenting 8% glucose and 4% xylose present in the same medium almost completely to ethanol in two days. On the other hand, Figure 4 shows that the parent yeast fusion 1400 (D'Amore, et al., 1989 and D'Amore, et al., 1990) can only ferment glucose, but not xylose, to ethanol. 1400 (pLNH32) (in short LNH32) and 1400(pLNH33) (in short LNH33) were developed by the transformation of the *Saccharomyces* fusion 1400 (D' Amore, et al., 1989 and D'Amore, et al., 1990) with two of the high-copy-number plasmids, pLNH32 and pLNH33, shown in Figure 1. To date, there have been four such high-copy-number plasmids reported, pLNH31, pLNH32, pLNH33, and pLNH34 (Ho and Tsao, 1995). Each of these plasmids can transform fusion 1400 to recombinant yeasts to co-ferment both glucose and xylose with similar efficiencies.

Yeasts 1400 (pLNH32), 1400(pLNH33), and related recombinant xylose-fermenting *Saccharomyces*, with their xylose metabolizing genes cloned on a 2μ-based stable high-copy-number plasmid, are quite suitable for a batch process fermentation. However, in a continuous process fermentation, after prolonged culture in a glucose-rich medium (more than 20 generations), 1400 (pLNH32), 1400(pLNH33), and similar plasmid-mediated recombinant yeasts lose their capability of fermenting xylose as shown in Figure 5 and Figure 6.

Generally, exogenous DNA or gene(s) can be cloned into yeasts by two separate ways. One way is to clone the exogenous DNA or gene(s) into a plasmid vector containing a selectable genetic marker and a functional yeast DNA replication origin or ARS (autonomous replicating sequence) (Struhl et al., 1979; Stinchcomb et al., 1980; Chan and Tye, 1980) that allows the plasmid to be able to replicate autonomously in its new host, followed by transformation of the desired yeast host with the plasmid containing the cloned DNA fragment or gene(s). The resulting yeast transformants are able to stably maintain the cloned gene in the presence of selection pressure. However, such cloned gene(s) are unstable after prolonged culture in non-selective medium (in the absence of selection pressure).

Another way to clone the exogenous DNA or gene(s) into a yeast host is to integrate the DNA or gene(s) into the yeast chromosome. In yeast, integrative transformation is almost always via homologous

recombination (Orr-Weaver, 1981). The simplest way to clone a desired gene into a yeast chromosome by integration is first to clone the desired gene into a plasmid which does not contain a replication of origin or ARS (autonomous replication sequences) but does contain a piece of the host DNA for targeting the integration to a specific site (Orr-Weaver, 1981). Transformation of the new yeast host with such an intact integrative vector will generate integrative transformants containing the desired gene cloned to the site next to the selected targeting yeast DNA sequences. However, the frequency of such integrative transformation is extremely low (1 to 10 transformants per µg DNA). Subsequently, it has been demonstrated that integrative vectors linearized within the DNA fragment homologous to the host chromosomal DNA can transform yeasts with much higher frequencies (100- to 1000-fold higher) (Orr-Weaver, 1981; Orr-Weaver and Szostak, 1983). It was suggested that double-stranded breaks, introduced by restriction enzyme digestion, are recombinogenic and highly interactive with homologous chromosomal DNA. This is particularly helpful for a complex plasmid, containing more than one yeast gene, so that one can direct the integration to a specific site by making a restriction enzyme cut within the corresponding region on the plasmid.

Another type of integration, also described as transplacment or gene disruption, makes use of double homologous recombination to replace yeast chromosomal DNA (Rothstein, 1981). Double homologous recombination vectors contain the exogenous DNA or gene(s) to be cloned and the selection marker, flanked by yeast DNA sequences

homologous to 5' and 3' regions of the segment of chromosomal DNA to be replaced. Prior to transformation, the vector is digested with restriction enzymes which liberate the transplacing fragment containing 5' and 3' ends homologous to the chromosomal DNA sequences at the desired integration sites. The latter strategy has become the method of choice for integrative transformation of yeast if a stable single-copy transformant is desired.

A number of strategies based on integration into reiterated chromosomal DNA have been used to generate stable multiple-copy integrants. For example, the delta sequence of yeast retrotransposon Ty (Sakai et al., 1990; Sakai et al., 1991), the highly conserved repeated sigma element (Kudla and Nicolas, 1992) and non-transcribed sequences of ribosomal DNA (Lopes et al., 1989; Lopes et al., 1991; Rossolini et al., 1992) have all been used as the target sites for multiple integration of exogenous gene(s) into yeast (Rothstein, 1991; Romanos et al., 1992).

Recent work reported in the literature on multiple integration of exogenous genes into the yeast chromosome has for the most part involved the use of either properly linearized non-replicative vectors or DNA fragments containing the desired gene(s) to be cloned and the genetic marker for selection, flanked with DNA sequences homologous to a region of yeast chromosomal DNA. Rarely, linearized replicative vectors and almost never intact replicative vectors, such as intact ARS vectors, were used to achieve such recombinant transformation. Thus,

since early work at the onset of developing yeast integrative transformation, (Szoatak and Wu (1979)), and despite the observation that DNA cloned on ARS vectors can integrate into the host chromosomes (Cregg et al., 1985; Kurtz et al., 1986), the use of intact ARS vectors (Struhl et al., 1979; Stinchcomb et al., 1980; Chan and Tye, 1980) for integration purposes has long since generally been abandoned. This has especially been true since the discovery that the double-stranded breaks introduced by restriction enzyme digestion are recombinogenic (Orr-Weaver, 1981; Orr-Weaver and Szostak, 1983).

In light of this background, there remain needs for more stable yeast which ferment xylose to ethanol, preferably xylose and glucose simultaneously to ethanol, and for facile and effective methods for making high copy number integrants. The present invention addresses these needs.

SUMMARY OF THE INVENTION

Accordingly, the present invention provides yeast containing multiple copies of stably cloned XR, XD, and XK genes, which even upon culture in non-selective medium for multiple generations (e.g. greater than 20) retain their full capability to ferment xylose to ethanol. More preferably, the XR, XD, and XK genes are all fused to promoters not inhibited by the presence of glucose and also not requiring the presence of xylose for their expression. Still more preferably, the yeast of the invention can co-ferment the two major constituents of cellulosic biomass, glucose and xylose, to ethanol.

Another embodiment of the present invention relates to the use of reiterated sequences, e.g. non-transcribed r-DNA sequences adjacent to the 5S DNA (Valenzuela et al., 1977), as homologous sequences for targeting high-copy-number integration of a DNA fragment containing XR, XD, and XK into the yeast genome via homologous recombination. For example, a replicative plasmid vector including the DNA fragment flanked by the homologous sequences can be used to target integration of the DNA fragment. A preferred method of the invention includes the steps of (a) transforming the cells with a replicative/integrative plasmid having exogenous DNA including a selection marker; and (b) repeatedly replicating the cells from step (a) to produce a number of generations of progeny cells while selecting for cells which include the selection marker (e.g. by replicating on selective plates), so as to promote the retention of the replicative and integrative plasmid in subsequent

generations of the progeny cells and the formation of progeny cells having multiple integrated copies of the exogenous DNA. In a further step, the cells from step (b) can be replicated to produce a number of generations of progeny cells in the absence of selection for cells which include the selection marker, so as to promote the loss of the plasmid in subsequent generations of progeny cells (thus leaving an enriched population of the stable integrants).

The invention also provides an advantageous mode for selection and maintenance of the desired transformants. It is well known that in minimal medium all microorganisms require the presence of a carbon source, such as glucose or xylose, for growth. However, most microorganisms do not require the presence of a carbon source for growth in rich medium. Nevertheless, the present invention provides the use a carbon source as the selection pressure for the selection of transformants even in rich medium, such as YEP (1% yeast extract plus 2% peptone). The development of stable transformants, such as 1400(LNH-ST) (Figure 7), which are capable of effective fermentation of xylose after culturing in non-selective medium for essentially unlimited generations, has been greatly facilitated by the discovery that many yeasts, particularly *Saccharomyces* yeasts, do naturally require the presence of a carbon source, such as xylose or glucose, for growth even in rich medium, as shown in Figure 8.

In a broad aspect, the invention also provides a method for integrating multiple copies of exogenous DNA

into reiterated chromosomal DNA of cells. The method includes (a) transforming the cells with a replicative and integrative plasmid having exogenous DNA including a selection marker. The method also includes (b) replicating the cells from step (a) to produce a number of generations of progeny cells while selecting for cells which include the selection marker, so as to promote the retention of the replicative and integrative plasmid in subsequent generations of the progeny cells and produce progeny cells having multiple integrated copies of the exogenous DNA. In a specific application, such a method includes (i) transforming yeast cells with a replicative plasmid having exogenous DNA including a selection marker, the exogenous DNA being flanked on each end by a DNA sequence homologous to a reiterated sequence of DNA of the host; (ii) repeatedly replicating the transformed yeast cells from step (i) to produce a number of generations of progeny cells while selecting for cells which include the selection marker, so as to promote the retention of the replicative plasmid in subsequent generations of the progeny cells and result in progeny cells each containing multiple integrated copies of the exogenous DNA; and (iii) replicating the progeny cells from step (ii) to produce a number of generations of progeny cells in the absence of selection for cells which include the selection marker, so as to promote the loss of the plasmid in subsequent generations of progeny cells and recover yeast cells each containing multiple copies of the exogenous DNA integrated into its chromosomal DNA.

In still another embodiment, the invention provides a yeast which ferments xylose to ethanol, the yeast

having multiple copies of exogenous DNA integrated into its chromosomal DNA. The exogenous DNA including genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase fused to non-glucose-inhibited promoters, wherein the yeast ferments glucose and xylose simultaneously to ethanol and substantially retains its capacity for fermenting xylose to ethanol for at least 20 generations even when cultured under non-selective conditions.

Another aspect of the invention relates to methods for fermenting xylose to ethanol, which include fermenting xylose-containing mediums with yeasts of the invention.

Another embodiment of the invention provides a plasmid vector for integrating an exogenous DNA sequence including a selection marker into chromosomal DNA of a target yeast cell. The inventive plasmid vector contains a functional yeast DNA replication origin and the exogenous DNA including the selection marker flanked on each end by a DNA flanking sequence which is homologous to a reiterated ribosomal DNA sequence of the target yeast cell. The plasmid further has a second selection marker in a position other than between the DNA flanking sequences.

A still further embodiment of the invention provides a plasmid vector for integrating an exogenous DNA sequence into a yeast to form stable integrants which ferment xylose to ethanol. The vector contains a functional yeast DNA replication origin and exogenous DNA

including genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase flanked on each end by a DNA flanking sequence which is homologous to a reiterated DNA sequence of the target yeast cell.

A still further aspect of the invention provides a method for forming cells having multiple integrated copies of an exogenous DNA fragment. This inventive method includes replicating cells having reiterated genomic DNA and which contain a replicative and integrative plasmid containing the exogenous DNA to produce multiple generations of progeny cells while selecting for cells which include the selection marker, so as to promote the retention of the replicative and integrative plasmid in subsequent generations of the progeny cells and produce progeny cells having multiple integrated copies of the exogenous DNA.

The invention provides yeasts containing stably cloned genes enabling their use under non-selective conditions (e.g. continuous fermentations) to coferment xylose and glucose to ethanol, while not losing their capacity to ferment xylose. In addition, the invention provides methods and materials for forming stable, multiple-copy integrants of yeast and other cells which are facile to perform and which can be controlled to modulate the number of copies of the integrated exogenous DNA. Additional embodiments, and features and advantages of the invention will be apparent from the following description and appended claims.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the restriction map of the plasmids pLNH31, -32, -33, and -34, and the genes cloned within.

Figure 2 shows that yeast transformant 1400(pLNH32) (in short LNH32) can effectively coferment glucose and xylose. The conditions used for culturing the yeast and for fermenting the sugars are similar to those described in Example 7.

Figure 3 shows that yeast transformant 1400 (pLNH33) (in short LNH33) can effectively coferment glucose and xylose. The conditions used for culturing the yeast and for fermenting the sugars are similar to those described in Example 7.

Figure 4 shows that the parent yeast fusion strain 1400 can ferment glucose but not xylose. The conditions used for culturing the yeast and for fermenting the sugars are similar to those described in Example 7.

Figure 5 demonstrates that yeast transformant 1400(pLNH32) (in short LNH32) with its xylose metabolizing genes cloned in the replicative plasmid pLNH 32 is not stable in a non-selective medium. After being cultured for 20 generations in a non-selective (for example, glucose) medium, 1400(pLNH32) lost its capability to ferment xylose.

Figure 6 demonstrates that yeast transformant 1400(pLNH33) (in short LNH33) with its xylose metabolizing genes cloned in the replicative plasmid pLNH 33 is not stable in a non-selective medium. After being cultured for 20 generations in a non-selective medium (for example, glucose medium), 1400(pLNH33) lost its capability to ferment xylose.

Figure 7 shows that yeast transformant 1400(LNH-ST) (in short LNH-ST) can stably maintain its xylose fermenting capability even after being cultured in non-selective medium for more than 40 generations.

Figure 8 demonstrates that *S. cerevisiae* and other *Saccharomyces* yeasts require a carbon source for growth even when rich media such as yeast extract and pepton were present in the medium. For example, these experiments showed that *S. cerevisiae* was unable to grow in the YEP medium containing 1% yeast extract and 2 % pepton, but was able to grow when glucose or xylulose was added to the YEP medium.

Figure 9A shows the restriction map of pLNH-ST, and the genes cloned within.

Figure 9B shows the genetic map (the order and orientation) of genes (5S rDNA, KK, AR, and KD) cloned in pLNH-ST. The oligonucleotides (for example, Oligo 25, Oligo 26, etc.) that are above or below the gene map are the primers used to characterize the order and orientation of the cloned genes by PCR.

Figure 10 is a schematic diagram outlining the construction of pBluescript II KS(-) containing the cloned XR, XD, XK genes: four such plasmids were constructed. The KK-AR-KD fragment cloned in pKS(-)-KK-AR-KD-3 was chosen to be cloned in pUCKm-rDNA(5S)-ARS for the construction of pUCKm-rDNA(5S)(KRD)-ARS, also designated as pLNH-ST.

Figure 11 shows that yeast transformant 1400(LNH-ST) (in short LNH-ST), superior to 1400(pLNH 32) and 1400(pLNH 33), can effectively coferment glucose and xylose. The conditions used for culturing the yeast and for fermenting the sugars are similar to those described in Example 7.

Figure 12 shows the genes cloned in and the restriction map of a broad-host plasmid for the isolation of ARS containing DNA fragments from the chromosome DNA of *S. cerevisiae* and other yeasts.

DETAILED DESCRIPTION OF THE INVENTION

For the purpose of promoting an understanding of the principles of the invention, reference will now be made to certain preferred embodiments thereof, and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the invention is thereby intended, such alterations, further modifications and applications of the principles of the invention as described herein are being contemplated as would normally occur to one skilled in the art to which the invention relates.

As mentioned above, one preferred aspect of the present invention provides recombinant yeasts incorporating stably cloned XR, XD and XK genes, which represent an improvement upon prior-reported recombinant yeasts. Generally, recombinant yeasts that can effectively coferment both glucose and xylose present in the same medium have been reported (Ho and Tsao, 1995). The yeasts made in this publication were accomplished by cloning properly modified XR, XD, and XK genes on a high copy number plasmid, pUCKm10, followed by using the resulting plasmid, pLNH3X (X=1 to 4) (Figure 1), to transform suitable natural yeasts. For example, the plasmids pLNH32 and pLNH33 were used to transform fusion yeast 1400 to 1400 (pLNH32) and 1400 (pLNH33), respectively. These recombinant *Saccharomyces* yeasts can effectively coferment both glucose and xylose present in the same medium to ethanol as shown in Figures 2 and 3, while the parent unengineered 1400 yeast can only ferment

glucose alone, not coferment both glucose and xylose (Figure 4).

Plasmid-mediated recombinant yeasts can maintain the cloned genes in the presence of selection pressure, but not in the absence of selection pressure. As demonstrated in Figures 5 and 6, 1400 (pLNH32) and 1400 (pLNH33) eventually lose their plasmids and their capability for fermenting xylose after prolonged culture in the absence of selection pressure.

It is highly desirable that recombinant industrial yeasts, particularly those strains used for the production of large volume industrial products, such as ethanol, be stable without requiring the presence of selection. The development of recombinant yeasts containing integrated XR, XD, and XK genes, as in the present invention, provides such stability. In addition, for the resulting recombinant yeasts to have the ability to coferment glucose and xylose at efficiencies similar to or better than 1400(pLNH32) and 1400(pLNH33), the recombinant yeasts must contain not only the integrated xylose metabolism genes, but also high numbers of copies of such integrated genes. In preferred aspects of the present invention, high-copy-number (hcn) integrants of yeasts (i.e. yeasts having at least about 10 integrated copies of the exogenous DNA) have been developed by targeting a non-coding region, such as a non-coding region of 5S ribosomal DNA (rDNA) as the site for multiple integration.

rDNA provides an advantageous location for integration because it is highly conserved, and yeasts generally contain more than 100 copies of the rDNA repeated sequences. It will be understood, however, that to achieve yeasts of the present invention, it will not be necessary to achieve integration of the desired genes at every occurrence of a repeated or reiterated sequence. It will be sufficient to achieve such integration at each of multiple sites of a reiterated sequence, i.e. two or more sites, in accordance with the broad aspects of the present invention.

In order to integrate hcn XR, XD, and XK into the yeast chromosome at the site of 5S rDNA, the integration plasmid, pLNH-ST, as shown in Figure 9, was constructed. pLNH-ST is a yeast-*E. coli* shuttle vector and a derivative of pUCKm 6 plasmid (Ho et al., 1984). The 5S rDNA sequences was inserted at the Xho I restriction site of pUCKm 6. The 5S rDNA sequence was copied from the yeast chromosomal DNA by the PCR technique and modified by the site-specific mutagenesis technique to add an XhoI restriction site in its center (approximately) sequence as shown in Figure 9. The XhoI fragment from pKS(-)-KK-AR-KD (Figure 10) (Ho and Tsao, 1995) has been inserted into the XhoI site of the 5S rDNA cloned in pLNH-ST.

pLNH-ST differs from other traditional 5S rDNA-based hcn yeast integrating vectors in that it also contains a functional yeast ARS sequence (Struhl et al., 1979; Stinchcomb et al., 1980; Chan and Tye, 1980) as shown in Figure 9. Thus, pLNH-ST is both a replicative vector and an integrative vector. Uniquely, pLNH-ST functions first

as a replicative vector then as an integrative vector in the development of recombinant yeasts containing high copies of integrated XR, XD, and XK. The ARS fragment was inserted at the EcoR1 site of pUCKm 6. In addition, pLNH-ST also contains the kanamycin resistance gene (Km^R) and the ampicillin resistance gene (Ap^R). Km^R functions as a geneticin resistance gene in yeasts and will confer its yeast transformants resistant to geneticin. The XhoI site of Km^R was removed by PCR technique without affecting its activity. Both Km^R and Ap^R are part of the original pUCKm 6 plasmid.

As noted above, the above-described vectors differ from those used in state-of-the-art techniques by containing an ARS sequence. In addition, in prior-reported methods for making hcn yeast integrants, integration of the cloned genes has taken place instantly, at the moment when the yeast cells are transformed with the exogenous genes. To the contrary, in accordance with preferred modes of the invention, integration of the cloned genes continues to take place gradually, long after transformation has been completed. In particular, transformation is established first via the presence of replicative plasmid, such as pLNH-ST, in the transformed yeast cells, and integration takes place only gradually via repeated replication of the transformants on plates containing selective medium.

Thus, this invention relates the use of the following procedures to develop yeast or other cell transformants containing hcn integrated cloned gene(s).

Host cells which contain reiterated DNA sequences, for example yeast or eukaryotic cells, are transformed with a replicative/integrative plasmid, such as pLNH-ST, and transformants containing high-copy numbers of the replicative/integrative plasmid are selected. The resulting selected transformants are repeatedly replicated onto fresh selective plates and grown to high cell density for a sufficient number of times to integrate the desired number of copies of the exogenous DNA, followed by culturing the transformants in non-selective medium for a sufficient number of generations to remove the replicative/integrative plasmids from the transformants. The resulting transformants can then be cultured in selective medium, and those transformants retaining their capability to effectively grow in selective medium will be those that contain hcn of the desired exogenous genes integrated into the chromosome of the yeast or other host cells. For example, fusion 1400 yeast has been transformed with pLNH-ST according to the above described procedures, and the resulting stable recombinant yeast, 1400(LNH-ST), can coferment both glucose and xylose better than 1400(pLNH 32) and 1400(pLNH33), as shown in Figure 11. Importantly, the newly-developed stable recombinant yeast, 1400(LNH-ST), can still ferment both glucose and xylose with equal efficiencies after being cultured in non-selective medium for 4, 20, and 40 generations as shown in Figure 7, while 1400(pLNH 32) and 1400(pLNH33) will lose most of their activity for fermenting xylose after 20 generations of being cultured in non-selective medium (Figures 5 and 6). Furthermore, 1400(LNH-ST) has subsequently been cultured in non-selective medium for several hundred generations,

and still retains its full activity in cofermenting both glucose and xylose.

In the preferred methods for developing stable hcn integrants, a common selection marker(s) is/are used for the selection and maintenance of both the plasmid-mediated activity and the activity contributed by the integrated genes with the same selective medium. In the present work, the common selection markers are the three cloned xylose metabolism genes, XR, XD, and XK, and the common selective medium is either rich or minimal medium (for yeasts) containing xylose. In addition, these cloned genes serve as the selection markers in rich medium for most *Saccharomyces* yeasts, since the applicants have shown that most of the *Saccharomyces* yeasts do require the presence of a carbon source, such as xylose, for growth even in rich medium (Figure 8). Although it is not crucial for yeasts chosen as hosts to require the presence of a carbon source in rich medium for growth, it is, nevertheless, much more convenient to be able to select the desired integrants on plates containing rich medium with xylose rather than on plates containing minimal medium with xylose. Preferred hosts for transformation in the present invention belong to the *Saccharomyces* species, since they are usually extraordinarily effective for fermenting glucose. In the event that species of yeasts desired for use as hosts for integrating high copy numbers of xylose metabolizing genes are found not to require the presence of a carbon source for growth in rich medium, a suitable mutant of that species which does require the carbon source in rich medium can be isolated using conventional procedures.

The replicative/integrative plasmid, such as pLNH-ST, for achieving hcn integration also desirably contains a second selection means for the selection of replicative plasmid-mediated transformants. For pLNH-ST, the second selection mechanism utilizes both Km^R and Ap^R as selectable markers. Although it is not crucial for a replicative/integrative vector to contain a second selection system, it will provide more preferred vectors, particularly if the ARS vector is not sufficiently stable even in the presence of the selection pressure, and the transformants have the tendency to lose most of their plasmids prior to integrating sufficient copies of the desired genes. When using vectors which contain a second selection mechanism, the transformants may be cultured in the presence of the second selective reagent to boost their plasmids' copy number, or to re-transform the transformants with the same vector but using the second selection mechanism to re-select the transformants so that the integration process can be continued or re-initiated.

The use of both Km^R and Ap^R as the second selection system is desirable for the applicants' preferred yeast. Km^R can be a dominant selection marker for transforming yeasts that are resistant to geneticin, but some yeasts are naturally resistant to geneticin without acquiring the plasmid containing Km^R. As a result, Km^R alone is not a preferred selection marker for the selection of yeast transformants. On the other hand, Ap^R can be effectively expressed in most yeasts, but it generally

cannot be used as a dominant selection marker for yeast transformation because most yeasts are naturally resistant to ampicillin. However, both Km^R and Ap^R together serve as an excellent dominant selection system for most yeasts, particularly the *Saccharomyces* yeasts. To use such a selection system, the transformants are first selected on plates containing YEPD (1% yeast extract, 2% peptone, 2% glucose) and proper concentrations of geneticin (20-80 μ g/ml, varying from species to species). The resulting transformants are screened for the expression of the Ap^R by the penicillinase test (Chevallier and Aigle, 1979) to identify true transformants.

The presence of Ap^R in pLNH-ST (Figure 9) and related replicative/integrative plasmids also serves another function. Since Ap^R is only present in the replicative plasmid and not present on the fragment integrated into the yeast chromosome, the ampicillin test also serves as a convenient process for identifying those transformants containing hcn integrated cloned genes but not plasmid vectors.

A feature of the inventive approach for providing stable recombinant yeasts containing hcn integrated gene(s) is that the number of copies of the gene(s) to be integrated can easily be controlled. For example, more copies of the XR-XD-XK genes can be inserted into the fusion yeast 1400 chromosome if another selection marker, such as Km^R, is inserted into the 5S rDNA fragment (or the targeting sequence). Furthermore, the inventive

methods for the development of hcn yeast integrants are also easier to accomplish than other reported approaches, wherein experimental conditions may have to be adjusted and controlled and the transformation process may have to be repeated before a stable strain could be obtained.

Thus, the applicants have improved upon the stability of prior recombinant xylose-fermenting yeasts, such as 1400 (pLNH32) and 1400 (pLNH33), and developed advantageously stable recombinant yeasts, for example 1400(LNH-ST), that will not require the presence of selection pressure to maintain the cloned genes and are also as effective as or even more effective for cofermenting glucose and xylose than 1400(pLNH32) and 1400(pLNH33). Furthermore, the applicants have also developed a convenient method that has provided the facile hcn integration of exogenous gene(s) into the cellular chromosome, wherein the number of copies of the gene(s) to be integrated is also readily controllable.

Similar to 1400 (pLNH32) and 1400(pLNH33), the preferred stable genetically engineered xylose-fermenting yeasts of the invention can also effectively coferment both glucose and xylose. This is because the XR, XD, and XK genes inserted into the chromosome of the new yeast hosts are all fused to intact 5' non-coding sequences from genes that can be efficiently expressed in yeast, encoding the production of high levels of enzymes, and also which are not inhibited by the presence of glucose in the medium. For example, the intact 5' non-coding DNA sequences that contain all the genetic elements for efficient expression of the glycolytic genes and for the

production of high levels of glycolytic enzymes are suitable as replacements for the intact 5' non-coding sequences of XR, XD, and XK for these purposes.

The XR, XD, and XK cloned on pLNH-ST are from *Pichia stipitis* (XR and XD) and *Saccharomyces cerevisiae*. (XK). However they can be from any microorganisms as long as they can produce high levels of the respective enzymes after they have been fused to the proper 5' non-coding sequences containing effective promoters, ribosomal binding sites, etc. For example, these three genes are well known to occur in a wide variety of microorganisms and numerous XR, XD and XK genes have been identified and isolated. The particular source of these genes is thus not critical to the broad aspects of this invention; rather, any DNAs encoding proteins (enzymes) having xylose reductase activity (the ability to convert D-xylose to xylitol with NADPH and/or NADH as cofactor), xylitol dehydrogenase activity (the ability to convert xylitol to D-xylulose with NAD^+ and/or $NADP^+$ as cofactor), or xylulokinase activity (the ability to convert D-xylulose to D-xylulose-5-phosphate) will be suitable. These genes may be obtained as naturally-occurring genes, or may be modified, for example, by the addition, substitution or deletion of bases to or of the naturally-occurring gene, so long as the encoded protein still has XR, XD or XK activity. Similarly, the genes or portions thereof may be synthetically produced by known techniques, again so long as the resulting DNA encodes a protein exhibiting the desired XR, XD or XK activity.

As examples, suitable sources of XR and XD genes include xylose-utilizing yeasts such *as Candida shehatae, Pichia stipitis, Pachysolen tannophilus,* suitable sources of XK genes include the above-noted xylose-utilizing yeasts, as well a xylose non-utilizing yeasts such as those from the genus *Saccharomyces,* e.g. *S. cerevisiae,* the genus *Schizosaccharomyces,* e.g. *Schizosaccharomyces pombe,* and bacteria such as *Escherichia coli,* Bacillus species, *Streptomyces* species, etc. Genes of interest can be recovered from these sources utilizing conventional methodologies. For example, hybridization, complementation or PCR techniques can be employed for this purpose.

A wide variety of promoters will be suitable for use in the invention. Broadly speaking, yeast-compatible promoters capable of controlling transcription of the XR, XD or XK genes will be used. Such promoters are available from numerous known sources, including yeasts, bacteria, and other cell sources. Preferably, the promoters used in the invention will be efficient, non-glucose-inhibited promoters, which do not require xylose for induction. In this regard, an "efficient" promoter as used herein refers to a 5' flanking sequence which provides a high level of expression of the fused gene. Promoters having these characteristics are also widely available, and their use in the present invention, given the teachings herein, will be within the purview of the ordinarily skilled artisan, as will be the fusion of the promoters to the XR, XD and XK genes, the cloning of the

promoter/gene fusion products into appropriate vectors and the use of the vectors to transform yeast. All of these manipulations can be performed using conventional genetic engineering techniques well known to the art and literature.

The yeast DNA replication origin, e.g. the ARS containing DNA fragment, can be obtained from yeast chromosomal DNA or from chromosomal DNA of other organisms, so long as the DNA fragment can function as an active replication origin to support autonomous replication of plasmid in the host chosen for hcn integration. DNA fragments which function as ARSs can readily be isolated by incorporating randomly-digested DNA fragments into an *E. coli* plasmid, followed by transformation of the desired host organism, e.g. a *Saccharomyces yeast,* with the resulting bank of plasmids, as reported in the literature (Stinchcomb et al., 1980; Ho et al., 1984).

Novel methods have been used to create the stable strains of the present invention. Nevertheless, there are several lines of evidence indicating that the cloned genes are not on a replicative plasmid and have been integrated into the host genome. For example, chromosomal DNA isolated from 1400(LNH-ST) can be used as template for the isolation of the cloned genes, including the fusions containing both the 5s rDNA and the cloned gene sequences, by the polymerase chain reaction (PCR). Also, while few plasmids (pLNH-ST) can be recovered from 1400(LNH-ST) via transformation of *E. coli* (Ward, 1990), under the same conditions, hundreds of pLNH32 or pLNH33

plasmids can be recovered from 1400 (pLNH32) and 1400(pLNH33), respectively. Furthermore, the initial 1400 fusion yeast transformants containing high copy numbers of the replicative plasmid pLNH-ST are unstable (with respect to their capability to ferment xylose) but positive for penicillinase (enzyme encoded by Ap^R) test (Chevallier and Aigle, 1979). On the contrary, the final stable transformants, 1400(LNH-ST), which retain their capability for fermenting xylose without the presence of selection, are found to be negative for penicillinase test. This is expected if the exogenous DNA is integrated at the site of 5S rDNA since Ap^R is not part of the DNA fragment to be integrated into the host chromosome. It is also possible that some of the stable yeast transformants may contain exogenous genes integrated at the ARS sites of the yeast chromosome.

For purposes of promoting a further understanding of the present invention and its features and advantages, the following Examples are provided. It will be understood, however, that these Examples are illustrative, and not limiting, of the invention.

EXAMPLE 1
SYNTHESIS OF THE 5S rDNA FRAGMENT BY PCR

For the synthesis of the 5S rDNA fragment by PCR (to serve as the yeast DNA sequence for targeting high-copy-number integration into the yeast chromosome), the following oligonucleotides were synthesized and used as the primers for PCR reactions according to the published 5S rDNA sequence (Valenzuela et al., 1977). In addition

to the 5S rDNA sequence, additional nucleotides specifying the Sal I restriction site were also added to the 5' terminal of primers to facilitate the cloning of the PCR synthesized 5S rDNA into an *E. coli* plasmid.

Oligonucleotide I: TTAGTCGACGTCCCTCCAAATGTAAAATGG.

Oligonocleotide II: AATGTCGACGTAGAAGAGAGGGAAATGGAG

Chromosomal DNA isolated from fusion yeast 1400 was used as the template for the PCR reaction. The PCR synthesized 5S rDNA fragment was first cloned into the *E.coli* pBluescript II KS(-) plasmid (Stratagene Cloning Systems, La Jolla, CA) at its SalI site. The resulting plasmid was designated as pKS-rDNA(5S).

EXAMPLE 2

INSERTION OF XHOI SITE INTO CLONED 5S rDNA SEQUENCE

The nucleotide sequence between -29 and -56 of the 5S rDNA sequence (Valenzuela et al., 1977) was modified by oligonucleotide-mediated site-specific mutagenesis (Kunkel, 1985; Kunkel et al., 1987). As a result, an XhoI restriction site was inserted at the specific site described above. The protocol provided by Bio-Rad Laboratories, Inc. for oligonucleotide-mediated site-specific mutagenesis was followed to accomplish this task, except that pKS plasmid was used rather than plasmid pTZ18U or pTZ19U. The resulting plasmid containing the mutated 5S rDNA was designated as pKS-5S rDNA(XhoI). The following oligonucleotide was used to

carry out the site-specific mutagenesis: GAGGGCAGGCTCGAGACATGTTCAGTAGG.

EXAMPLE 3

ISOLATION OF DNA FRAGMENTS FROM *S. CEREVISIAE* DNA OR OTHER DNA FUNCTIONING AS ARS IN YEASTS

S. cerevisiae DNA (or DNA from other yeasts or other organisms) was digested with Sau3A restriction enzyme and cloned into the Bam H1 site of pUCKm6 (Figure 12) (Ho, et al., 1984). The resulting bank of plasmids was used to transform *S. cerevisiae*. Those transformants that were capable of growing on plates containing YEPD (1% yeast extract, 2% peptone, and 2% glucose) and 50 µg/ml geneticin and which were also positive for the penicillinase test (Chevallier and Algle, 1979) were selected. The plasmids from the selected true transformants were recovered by a procedure similar to that described by Ward (1990).

The yeast DNA fragments inserted in pUCKm6 (Figure 12) and recovered from the yeast transformants should all contain a segment of DNA that can function as an ARS (autonomous replicating sequence) in *S. cerevisiae*, possibly in other yeasts as well. The DNA inserts were digested with various restriction enzymes and the resulting DNA fragments were re-inserted into pUCKm6. The latter plasmids were used to retransform *S. cerevisiae*. Any properly-sized restriction fragments that can make pUCKm6 function effectively as a yeast plasmid must contain an effective "ARS" and can be used

to construct replicative/integrative vectors such as pLNH-ST for high-copy-number integration of exogenous gene(s) into the chromosomes of *S. cerevisiae*. These restriction fragments are also likely to function as ARS's in other yeasts, and are suitable for the construction of replicative/integrative plasmids for other yeasts.

EXAMPLE 4

REMOVAL OF THE XHOI RESTRICITON SITE FROM THE GENETICIN (KANAMYCIN) RESISTANCE GENE, KM^R

The geneticin (kanamycin) resistance gene, Km^R, from Tn 903 (A. Oka et al., 1981) and the 5S rDNA fragment described in Example 1 are part of the plasmid designed for the integration of multiple copies of exogenous genes into the yeast chromosome. However, Km^R contains an XhoI site in its coding sequence. This is in conflict with the fact that an XhoI site has been engineered into the center of the cloned 5S rDNA sequence to be used for inserting exogenous genes such as XR, XD, and XK into the plasmid for integration. Thus, it is necessary to remove the XhoI site from Km^R. This can be accomplished by a number of different approaches. The applicants chose to use site-specific mutagenesis by the overlap extension PCR technique (S. N. Ho, et al., 1989) to remove the XhoI site from Km^R without changing its amino acid coding sequence and without affecting the catalytic activity of the enzyme encoded by the gene. The Km^R gene cloned in pUCKm6 (Figure 12) was converted to Km^R(-Xho) as described above.

The four oligonucleotides used to accomplish this task are listed below.

Oligonucleotide I: GGCCAGTGAATTCTCGAGCAGTTGGTG

Oligonucleotide II: TGGAATTTAATCGCGGCCCCTAGCAAGACG

Oligonucleotide III: TTACGCCAAGCTTGGCTGC

Oligonucleotide IV: TTCAACGGGAAACGTCTTGCTAGGGGCCGC

pUCKm6 (Figure 12) is a derivative of pUC9. Part of Oligo I and the entire Oligo III are synthesized according to the sequence of the polylinker region of pUC9 (Sambrook, et al. 1989).

The above-described genetic manipulation of pUCKm6 not only resulted in the deletion of the XhoI restriction site from the coding region of Km^R but also inserted an XhoI restriction site between the Km^R coding sequence and the EcoRI site of pUCKm6. The resulting plasmid was designated as pUCKm(-XhoI)(+XhoI). The addition of an XhoI site downstream to the Km^R coding sequence is to facilitate the insertion of the 5S rDNA fragment described in Example 1 into the newly developed plasmid pUCKm(-Xho)(+Xho).

EXAMPLE 5
CONSTRUCTION OF PLASMID pLNH-ST

The plasmid pUCKm(-XhoI)(+XhoI) described in Example 4 was used for the construction of pLNH-ST, shown in

Figure 9. First, the Sal I fragment containing the 5S rDNA(XhoI) was isolated from pKS-5S rDNA(XhoI) and inserted at the XhoI site of pUCKm(-XhoI)(+XhoI). The resulting plasmid was designated as pUCKm-rDNA(5S). To the latter plasmid, an EcoRI fragment containing an effective ARS isolated from *S. cerevisiae* (according to the procedure described in Example 3) was inserted into the EcoRI site of pUCKm-5S rDNA, and the resulting plasmid was designated as pUCKm-5S rDNA-ARS. To the latter plasmid, the XhoI fragment from pKS(-)-KK-AR-KD-3 containing the cloned XR, XD, and XK fused to yeast alcohol dehydrogenase promoter (XR), and pyruvate kinase promoter (for both XD & XK), were inserted into the XhoI site located at the center of the cloned 5S rDNA sequence. The resulting plasmid, pUCKm-rDNA(5S)(KDR)-ARS, also designated pLNH-ST, shown in Figure 9.

EXAMPLE 6

TRANSFORMATION OF FUSION YEAST 1400 WITH PLNH-ST AND SELECTION OF STABLE TRANSFORMANTS 1400 (LNH-ST)

pLNH-ST was used to transform fusion strain 1400 by electroporation under the conditions used for transformation of strain 1400 by plasmids pLNH32 and pLNH33 (International Publication No. 95/13362, May 18, 1995, publishing International Application No. PCT/US94/12861, filed November 8, 1994). Briefly, fifty ml yeast cells, grown to early log phase (Klett Unit (KU) 140-190), were centrifuged to remove the medium, washed twice with cold water, once with cold 1 M sorbitol, and resuspended in 200 µl 1 M sorbitol. Sixty µl of the cells were transferred into a 4 ml presterilized plastic

tube (with cap) and to which 1 µg plasmid DNA was added. Fifty µl of the resulting cells and plasmid mixture were pipetted into a precooled gene pulser cuvette with a 0.2 cm electrode gap and the content in the cuvette was subjected to pulse by the gene pulser with a pulse controller (BioRad) at 2.0 KV, 25 µF, 200 ohms.

Immediately, .50 ml YEPD was added to the cuvette. The content of the cuvette was transferred to a new 4 ml sterilized plastic tube and incubated at 30°C for 1 hr. 100 µl of the cells were plated on agar plates containing YEPD and 40 µg/ml G418 (geneticin). Fast growing colonies were selected and replicated on another plate containing the same medium. The selected colonies were subjected to the ampicillin test (Chevallier and Aigle, 1979) until a positive one was identified. The above-described electroporation procedure is based on that reported by Becker and Guarente (1971).

Once a transformant had been positively identified by the penicillinase test, it was maintained on a YEPX (1% yeast extract, 2% peptone, 2% xylose) plate. Initially, the transformants were very unstable. They lost their xylose fermenting capability if cultured in YEPD medium over 20 generations. However, by continuing to culture the transformants to stationary phase on YEPX plates, and repeatedly transferring them to fresh YEPX plates, the transformants gradually became stable with regard to their capability to ferment xylose. Once stable, the transformants could be cultured in non-selective medium for several hundred or more generations

and were still capable of co-fermenting both glucose and xylose, as demonstrated in Example 8.

EXAMPLE 7

CO-FERMENTATION OF GLUCOSE AND XYLOSE WITH 1400 (LNH-ST)

A mixture of glucose and xylose (approximately 10% glucose and 5% xylose) was fermented by strain 1400 (LNH-ST) under the conditions described below. The seed cultures of 1400 and 1400 (LNH-ST) were cultured aerobically in liquid YEPD medium until mid-log phase (between 400-450 Klett Units (KU)) and stored at 4°C. New seed cultures were prepared once a month by transferring 2 ml of the culture to 50 ml of fresh YEPD and cultured as described above. 2 ml of the seed cultures of 1400 (LNH-ST) were inoculated into 100 ml of YEPD medium in a 300 ml Erlenmeyer flask equipped with a side-arm which allowed direct monitoring of the growth of the yeast cultures by the Klett colorimeter. The culture was incubated in a shaker at 30°C and 200 rpm aerobically.

When the cell density reached mid-log phase (400-450 KU), 20 ml (50%) glucose and 10 ml (50%) xylose were added to the flask. After thorough mixing, 1 ml of the culture mixture was removed from the flask to serve as the zero sample. The flask was then sealed with Saran wrap to allow fermentation to be carried out anaerobically. One ml samples of the fermentation broth were removed at proper intervals (every 6 hrs.) to serve as samples for measuring glucose, xylose, xylitol, and glycerol contents of the broth during fermentation by

HPLC as described in Example 9. The results, shown in Figure 11 , demonstrate that the genetically engineered yeast 1400 (LNH-ST) can co-ferment most of the 10% glucose and 5% xylose to ethanol in 30 hrs. The fermentation was carried out under normal conditions, without requiring special medium or pH, and also without requiring growth of yeast to high cell density. Thus, the genetically engineered 1400 (LNH-ST) can effectively co-ferment high concentrations of both glucose and xylose to ethanol with very little xylitol produced as a by-product. In comparison to the recombinant *Saccharomyces* 1400 (pLNH32) and 1400 (pLNH33) shown in Figures 2 and 3, 1400 (LNH-ST) co-fermented both glucose and xylose somewhat better than the two previously developed yeasts.

EXAMPLE 8

COMPARISON OF THE STABLE STRAIN 1400 (LNH-ST) WITH 1400 (LNH32) AND 1400 (LNH33) IN CO-FERMENTING GLUCOSE AND XYLOSE AFTER CULTURE IN NON-SELECTIVE MEDIUM FOR 4, 20, AND 40 GENERATIONS.

As described in Example 7, 2 ml each of the seed cultures of 1400 (LNH-ST), 1400 (LNH32), and 1400 (LNH33) were inoculated into 50 ml YEPD in separate 250 ml Erlenmeyer flasks equipped with side-arms. After the cells were cultured to 400-450 KU, 2 ml of the fresh culture from each flask were transferred to a new flask. This process was repeated 10 times for 1400 (LNH-ST) and 5 times for 1400 (LNH32) and 1400 (LNH33). The 1400 (LNH-ST) cultures that were cultured for 4, 20, and 40 generations in non-selective medium (each transfer being considered as four generations cultured in non-selective medium) were used to co-ferment glucose and xylose under

similar conditions described in Example 7. The fermentation samples were taken and analyzed identically as described in Example 7. Similarly, the 1400 (LNH32) and 1400 (LNH33) cultures that were cultured for 4 and 20 generations in non-selective medium were used to co-ferment glucose and xylose. Samples were again taken at proper intervals after fermentation was initiated for analysis by HPLC and compared in Figures 4 to 6. These results clearly demonstrate that 1400 (LNH-ST) is far more stable than 1400 (LNH32) and 1400 (LNH33) in maintaining its xylose fermenting capability after being cultured in non-selective medium for more than 40 generations.

EXAMPLE 9
HPLC Analysis of Fermentation Samples

The samples containing the fermentation broth (0.6 ml to 1.0 ml) removed from the cultures were kept in 1.5 ml Eppendorf tubes. The cells and other residues were removed by centrifugation in microfuge (topspeed) for 10 min. The supernatant was diluted 10 fold. The resulting diluted samples were analyzed for its ethanol, glucose, xylose, xylitol, and glycerol contents by high performance liquid chromatography (HPLC), using a Hitachi system according to the following conditions.

- Column: BioRad HPX-87H
- Mobile Phase: 0.005 M H_2SO_4
- Flow Rate: 0.8 ml/min.
- Detection: RI detector
- Temperature: 60°C

· Injection Volume: 20 µl

EXAMPLE 10

GENETIC CHARACTERIZATION OF CHROMOSOMAL DNA FROM THE STABLE TRANSFORMANTS 1400 (LNH-ST)

Based on the restriction and PCR analysis, the genetic map (the order and orientation) of the cloned genes, KK, AR, KD, and 5S rDNA present in pLNH-ST, have been determined as shown in Figure 9B. Experiments have been designed to determine whether these genes (KK, AR, and KD) have been integrated into the loci of the 5S rDNA. If these genes have been integrated into the yeast chromosome at the loci of the 5S rDNA as anticipated, the correct size of DNA fragments containing the following combination of partial or intact genes such as 5S rDNA-KK; 5S rDNA-KD; KK-AR, and AR-KD should have been obtained by using 1400 (LNH-ST) chromosomal DNA as the template and the oligonucleotides indicated on the genetic map (Figure 9B) as the primers to carry out DNA synthesis by PCR. If these genes have not been integrated into the yeast chromosome, no such combination of genes or gene fragments should have been obtained by the above described experiments. If these genes have been integrated elsewhere in the yeast chromosome rather than at the loci of 5S rDNA, some of the above described combination of genes or gene fragments should be obtained from the above described experiments, but not those containing the 5S rDNA fragment; such as 5S rDNA-KK and 5S rDNA-KD. For carrying out the above described experiments, chromosomal DNA was isolated from 1400 (LNH-ST), using the protocol provided by Qiagen, Chatsworth,

CA. Positive results were obtained from PCR synthesis by using the following pairs of primers (see Figure 9): Oligo 25 and Oligo 369; Oligo 26 and Oligo 369; Oligo 370 and Oligo 96; Oligo 97 and Oligo 99; Oligo 982 and Oligo27. Thus, based on these analyses, the DNA fragment containing KK-AR-KD seems indeed being integrated in the 1400 yeast chromosome at its 5S rDNA loci.

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WHAT IS CLAIMED IS:

1. A yeast which ferments xylose to ethanol, comprising:

a yeast having genes integrated at each of multiple reiterated ribosomal DNA sites of the yeast, said genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase.

2. The yeast of claim 1 which also ferments glucose to ethanol.

3. The yeast of claim 2 which is *Saccharomyces*.

4. The yeast of claim 3 wherein said sites are non-transcribed DNA sites.

5. The yeast of claim 1 wherein the genes are fused to non-glucose-inhibited promoters and the yeast simultaneously ferments glucose and xylose to ethanol.

6. The yeast of claim 5 wherein the promoters do not require xylose for induction.

7. The yeast of claim 3 wherein the genes are fused to non-glucose-inhibited promoters and the yeast simultaneously ferments glucose and xylose to ethanol.

8. The yeast of claim 4 wherein the genes are fused to non-glucose-inhibited promoters and the yeast simultaneously ferments glucose and xylose to ethanol, the promoters also not requiring xylose for induction.

9. The yeast of claim 6 wherein the xylose reductase and xylitol dehydrogenase genes are from natural yeast which ferment xylose to ethanol.

10. The yeast of claim 9 wherein the natural yeast are *Candida Shehatae, Pichia stipitis* or *Pachysolen tannophilus*.

11. The yeast of claim 9 wherein the xyulokinase gene is from a yeast or bacteria.

12. The yeast of claim 11 wherein the xyulokinase gene is from *Candida Shehatae, Pichia stipitis, Pachysolen tannophilus, Saccharomyces cerevisiae, Schizosaccharomyces pombe*, or *Escherichia coli*.

13. The yeast of claim 1 having said genes integrated at at least about 10 ribosomal DNA sites of the yeast.

14. A method for integrating multiple copies of exogenous DNA into reiterated chromosomal DNA of cells, comprising:

(a) transforming the cells with a replicative and integrative plasmid having exogenous DNA including a first selection marker; and

(b) repeatedly replicating the cells from step (a) to produce a number of generations of progeny cells while selecting for cells which include the selection marker, so as to promote the retention of the replicative and integrative plasmid in subsequent generations of the

progeny cells and produce progeny cells having multiple integrated copies of the exogenous DNA.

15. The method of claim 14, wherein the plasmid DNA also includes a second selection marker for selecting cells which include the plasmid.

16. The method of claim 14 wherein the cells are yeast or eukaryotic cells, and wherein the method further includes the step of repeatedly replicating the progeny cells from step (b) to produce a number of generations of progeny cells in the absence of selection for cells which include the selection marker, so as to promote the loss of the plasmid in subsequent generations of progeny cells and recover yeast cells each containing multiple copies of the exogenous DNA integrated into its chromosomal DNA.

17. The method of claim 16 wherein the cells are yeast cells and the exogenous DNA includes genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase, which also serve as the first selection marker.

18. The method of claim 14, which comprises:

(i) transforming yeast cells with a replicative plasmid having exogenous DNA including a selection marker, the exogenous DNA being flanked on each end by a DNA sequence homologous to a reiterated sequence of DNA of the host;

(ii) repeatedly replicating the transformed yeast cells from step (i) to produce a number of generations of progeny cells while selecting for cells which include the

selection marker, so as to promote the retention of the replicative plasmid in subsequent generations of the progeny cells and result in progeny cells each containing multiple integrated copies of the exogenous DNA; and

(iii) replicating the progeny cells from step (ii) to produce a number of generations of progeny cells in the absence of selection for cells which include the selection marker, so as to promote the loss of the plasmid in subsequent generations of progeny cells and recover yeast cells each containing multiple copies of the exogenous DNA integrated into its chromosomal DNA.

19. Yeast cells produced by the method of claim 18.

20. The yeast cells of claim 19, wherein the exogenous DNA includes genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase, and the yeast cells ferment xylose to ethanol.

21. The yeast cells of claim 20, wherein said genes are fused to non-glucose-inhibited promoters which do not require xylose for induction, and wherein the yeast cells ferment glucose and xylose simultaneously to ethanol.

22. Yeast cells according to claim 21 which substantially maintain their capacity to ferment xylose to ethanol when cultured under non-selective conditions for at least 20 generations.

23. A yeast which ferments xylose to ethanol, comprising:

a yeast having multiple copies of exogenous DNA integrated into chromosomal DNA of the yeast, the exogenous DNA including genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase fused to non-glucose-inhibited promoters, the yeast fermenting glucose and xylose simultaneously to ethanol and substantially retaining its capacity for fermenting xylose to ethanol for at least 20 generations when cultured under non-selective conditions.

24. The yeast of claim 23, wherein said promoters do not require xylose for induction

25. A yeast which ferments xylose to ethanol, comprising:

a yeast having multiple copies of an introduced DNA containing genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase, the yeast fermenting xylose to ethanol and substantially retaining its capacity for fermenting xylose to ethanol when cultured under non-selective conditions for at least 20 generations.

26. The yeast of claim 25, wherein the promoters do not require xylose for induction

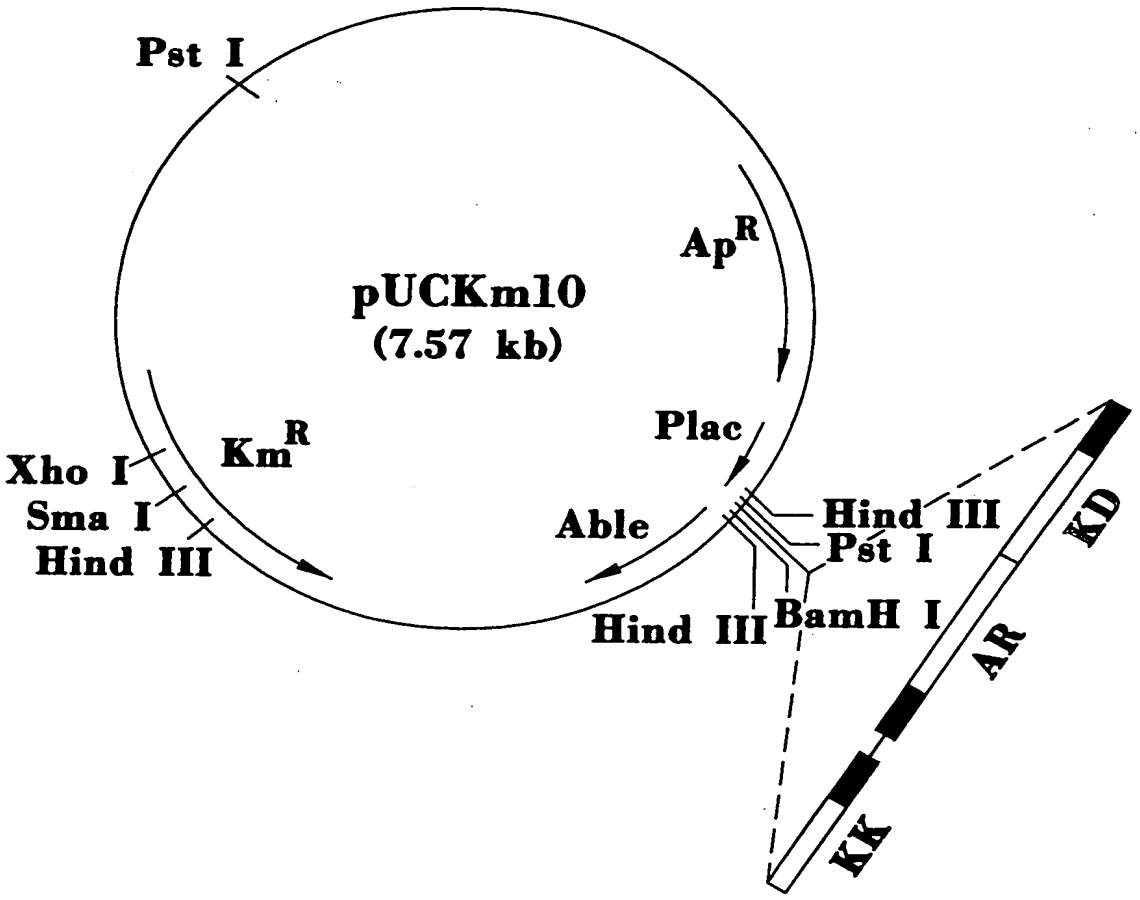
27. A method for fermenting xylose to ethanol, comprising fermenting a xylose-containing medium with a yeast of claim 1, 22, 23, 24, 25 or 26, to produce ethanol.

28. A plasmid vector for integrating an exogenous DNA sequence including a first selection marker into chromosomal DNA of a target yeast cell, the plasmid vector containing a functional yeast DNA replication origin and the exogenous DNA flanked on each end by a DNA flanking sequence which is homologous to a reiterated ribosomal DNA sequence of the target yeast cell, the plasmid further including a second selection marker in a position other than between the DNA flanking sequences.

29. A plasmid vector for integrating an exogenous DNA sequence into a yeast to form stable integrants which ferment xylose to ethanol, the plasmid vector containing a functional yeast DNA replication origin and exogenous DNA including genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase flanked on each end by a DNA flanking sequence which is homologous to a reiterated DNA sequence of the target yeast cell.

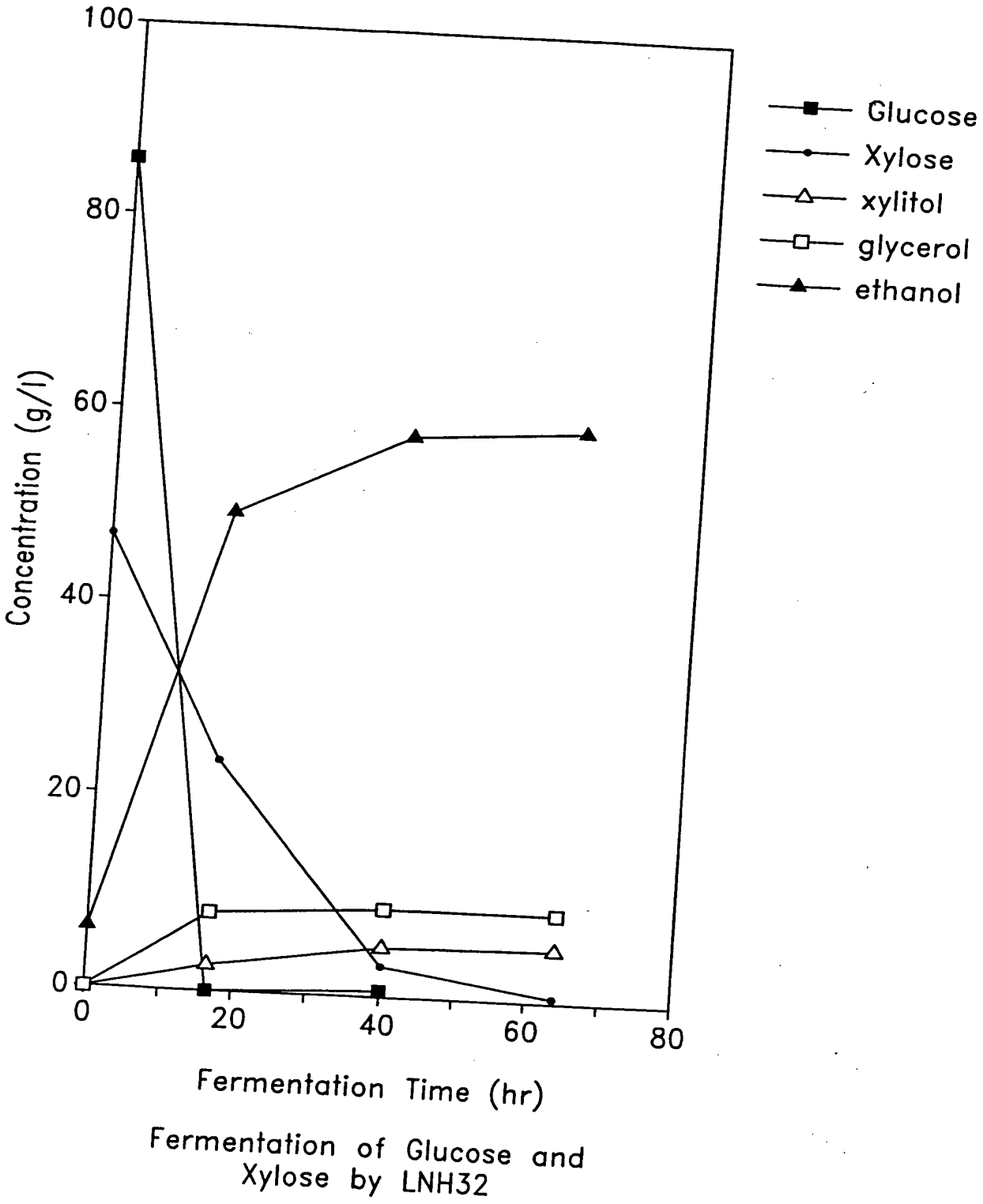
30. A method for forming cells having multiple integrated copies of an exogenous DNA fragment, comprising:

replicating cells having reiterated genomic DNA and which contain a replicative and integrative plasmid containing the exogenous DNA to produce multiple generations of progeny cells while selecting for cells which include the selection marker, so as to promote the retention of the replicative and integrative plasmid in subsequent generations of the progeny cells and produce progeny cells having multiple integrated copies of the exogenous DNA.



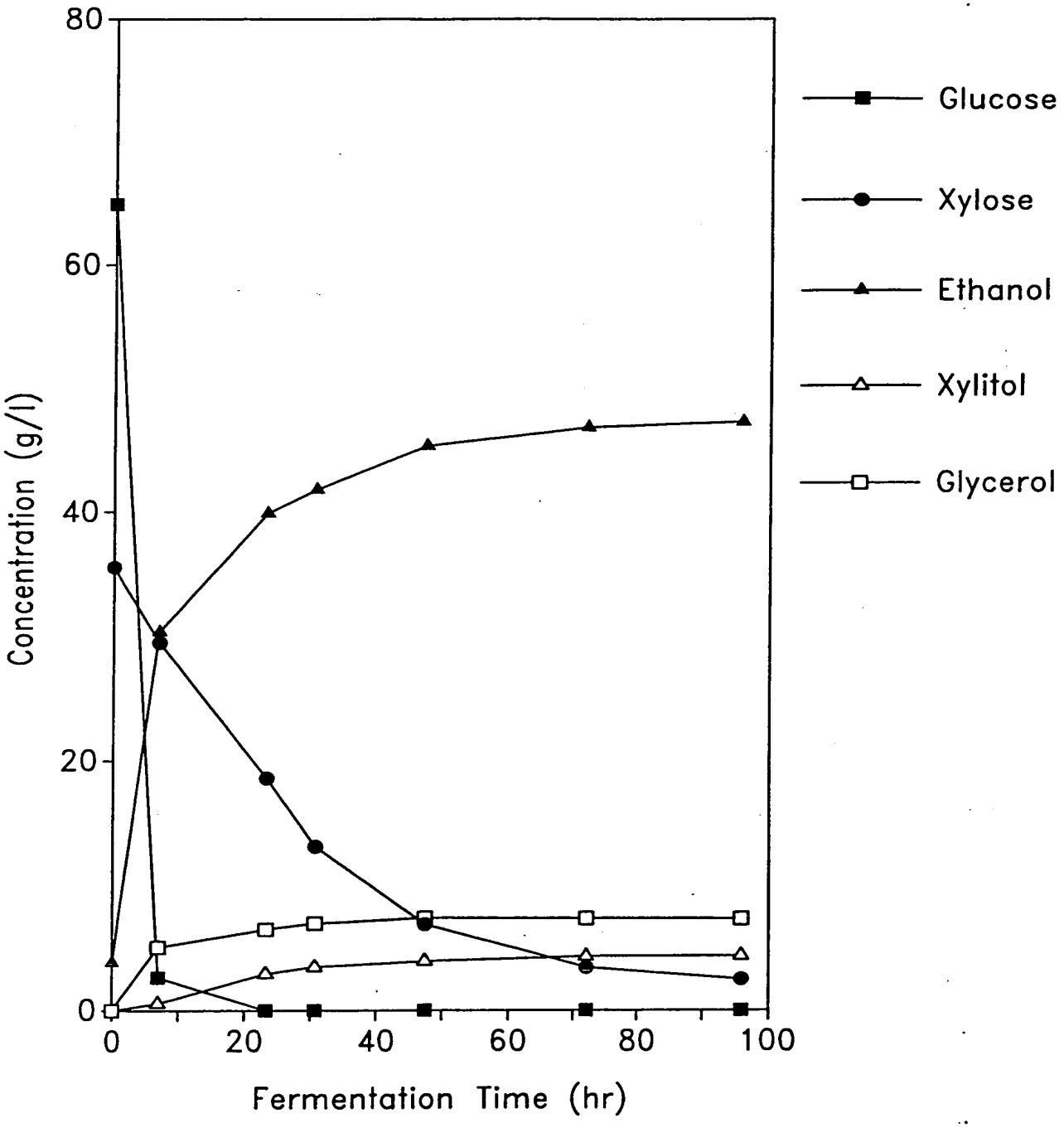
pLNH31, pLNH32, pLNH33, or pLNH 34

Fig. 1



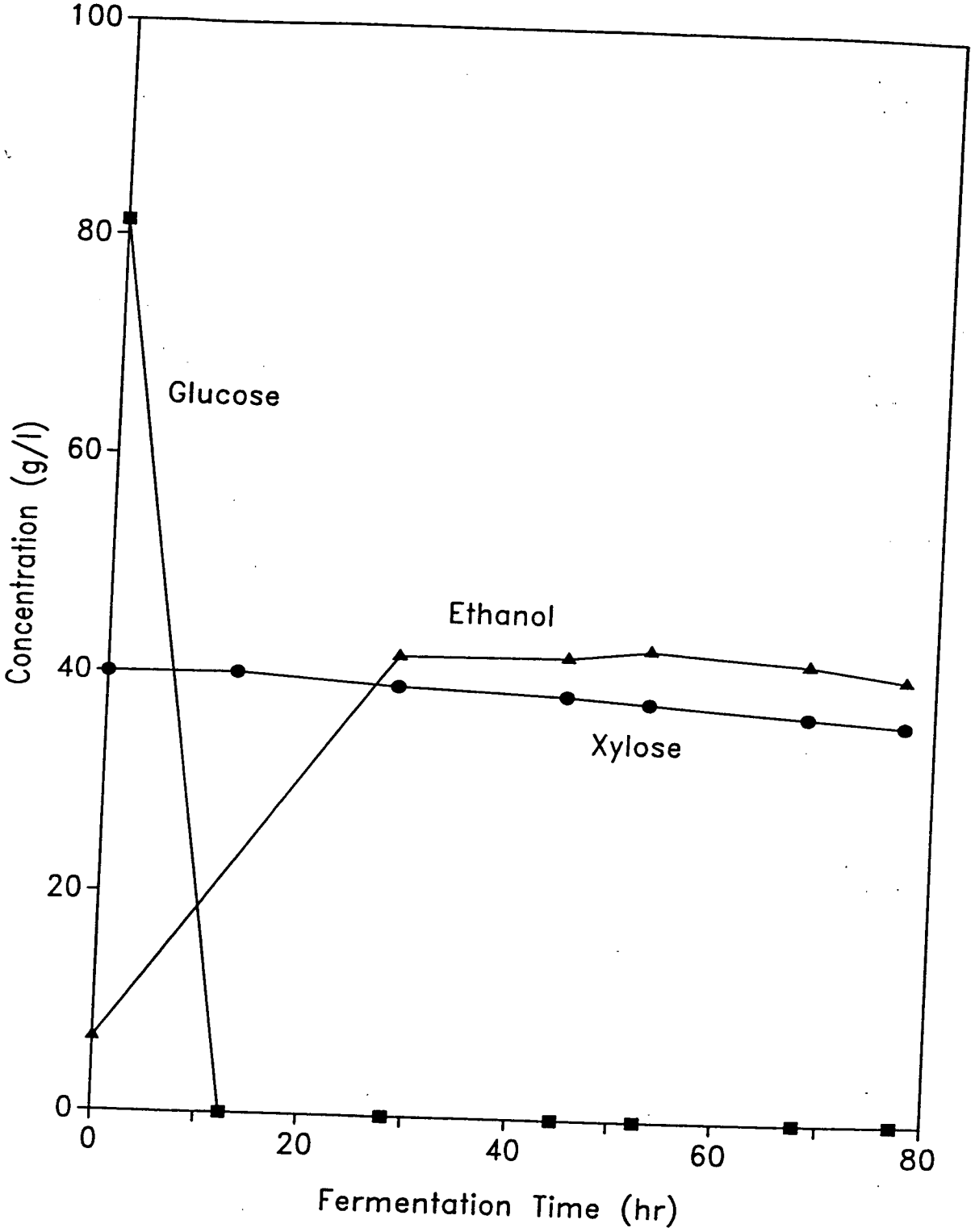
Fermentation of Glucose and Xylose by LNH32

Fig. 2



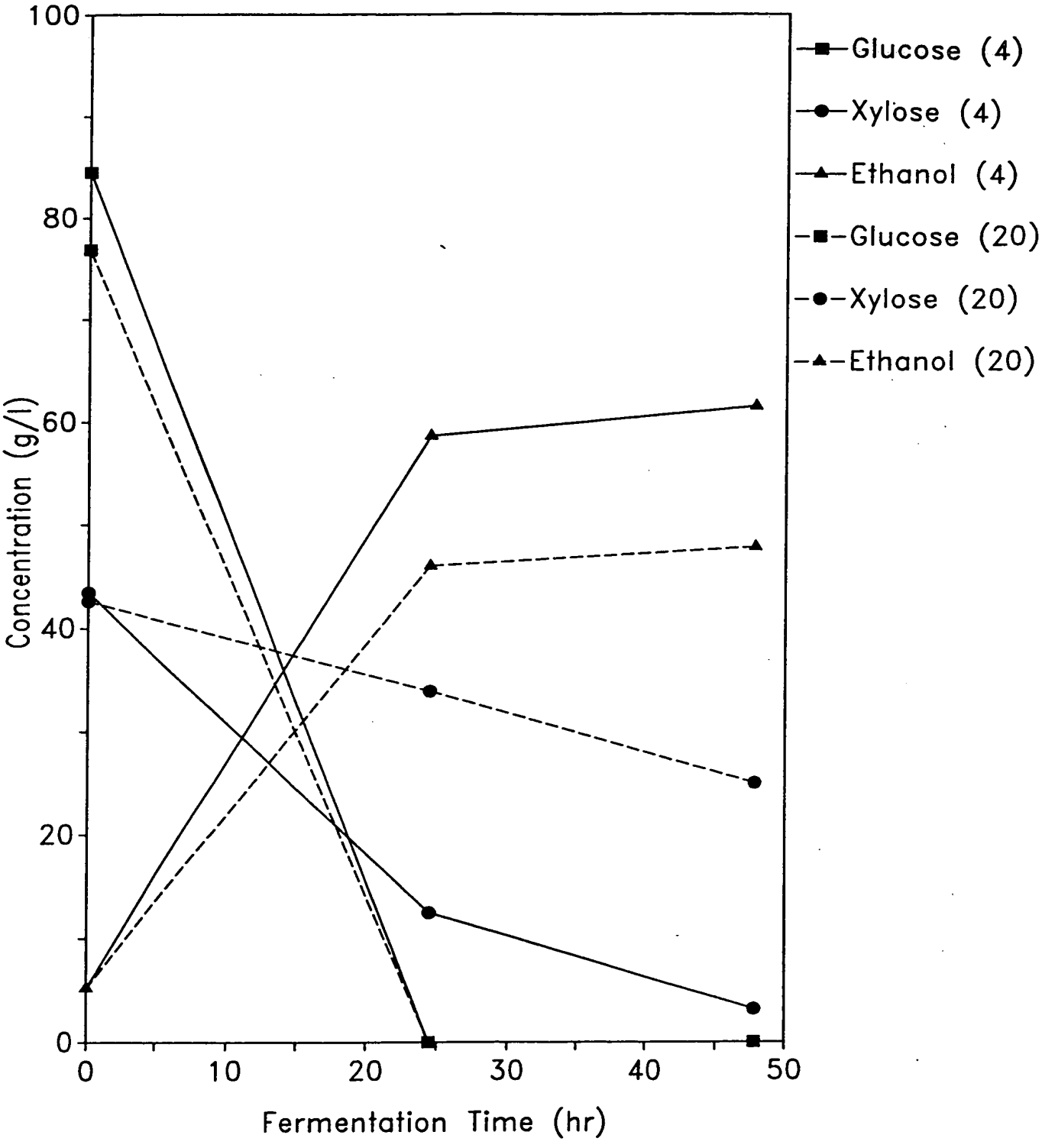
Simultaneous Fermentation of Glucose and Xylose by Recombinant Saccharomyces LNH33

Fig. 3



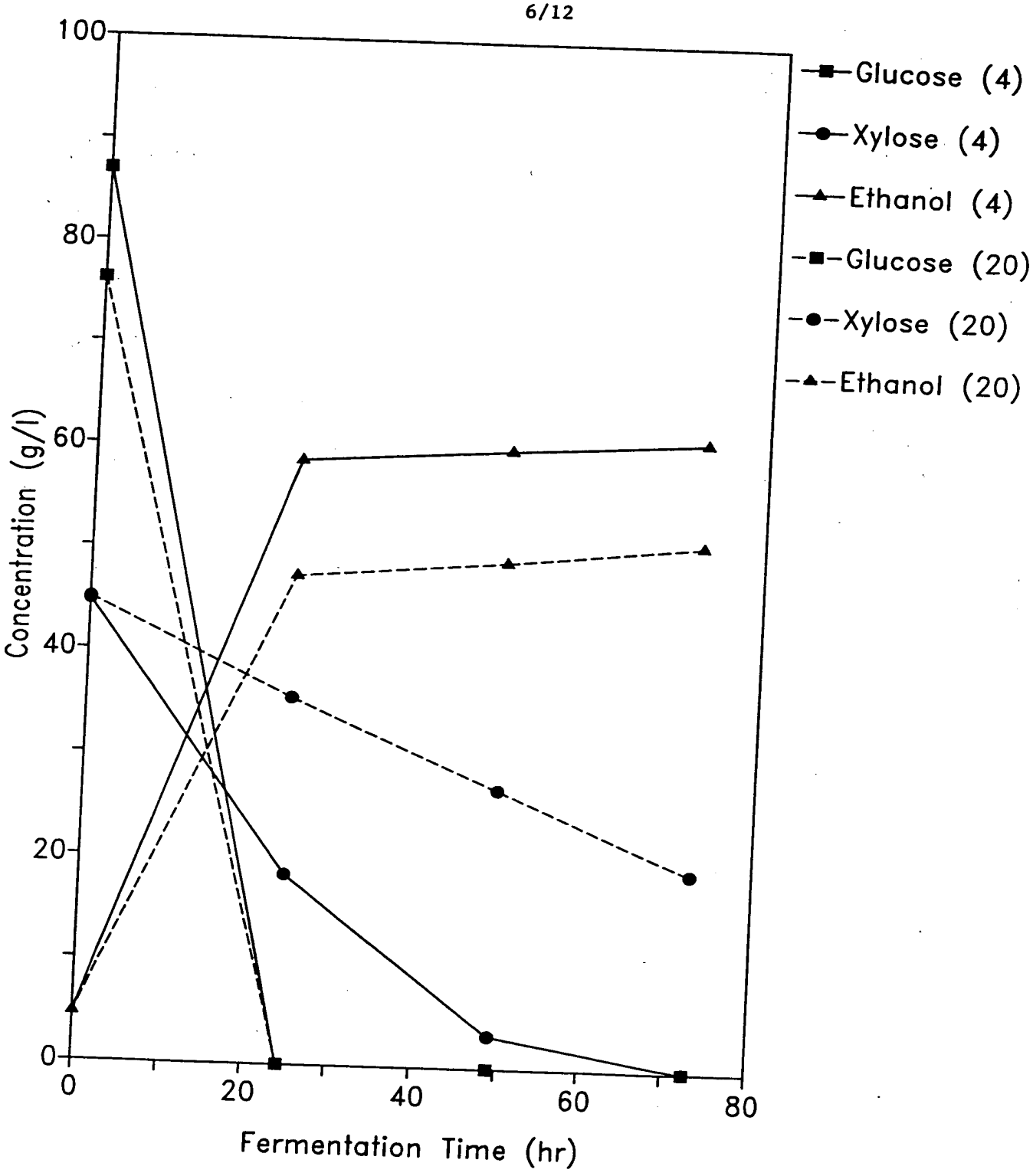
Fermentation of Glucose and Xylose by the Un-Engineered Parent 1400 *Saccharomyces* Yeast

Fig. 4



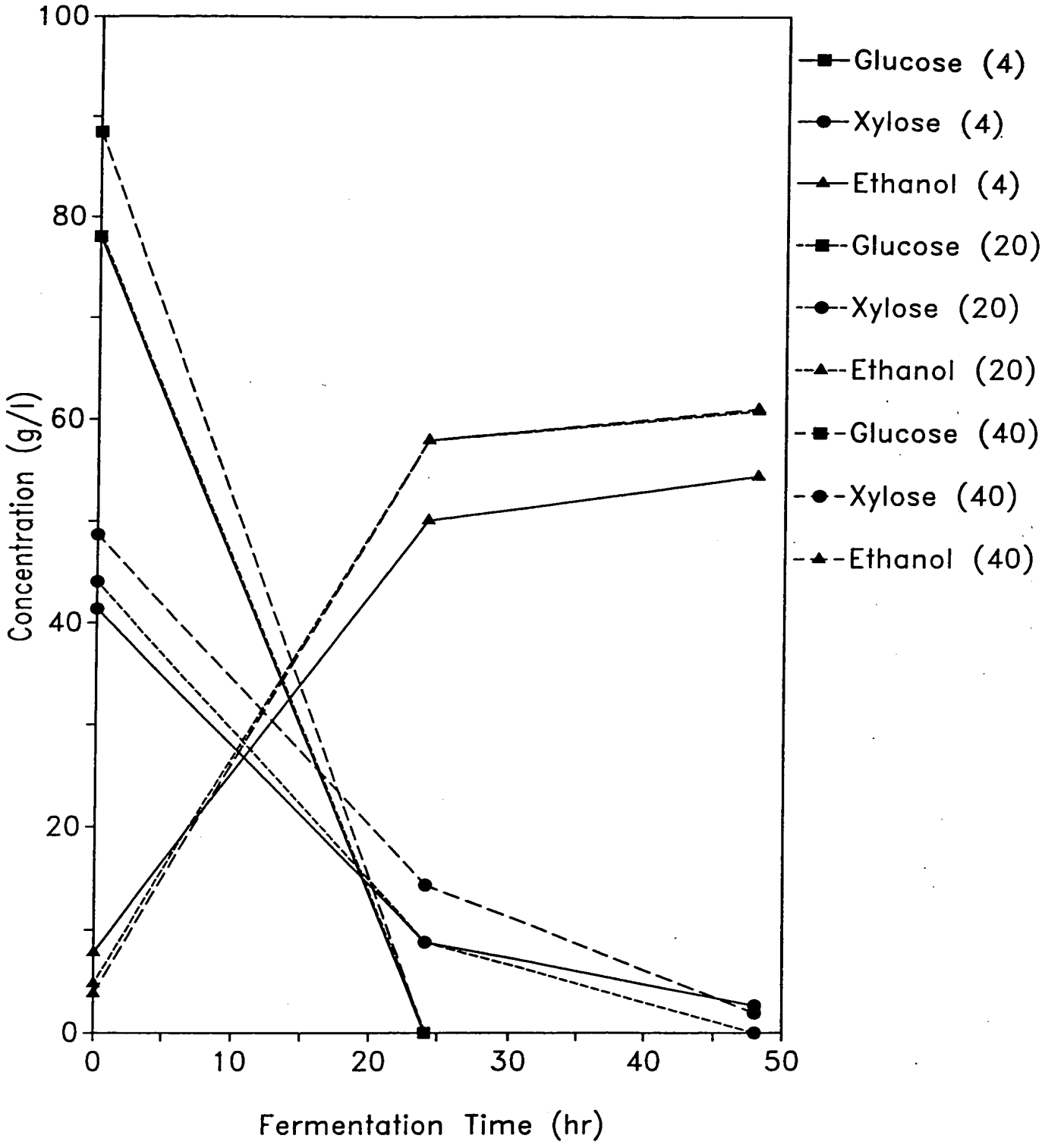
Fermentation of glucose and xylose by LNH32 after being cultured for 4 and 20 generarions in non-selective (glucose) medium.

Fig. 5



Fermentation of glucose and xylose by LNH33 after being cultured for 4 and 20 generations in non-selective (glucose) medium.

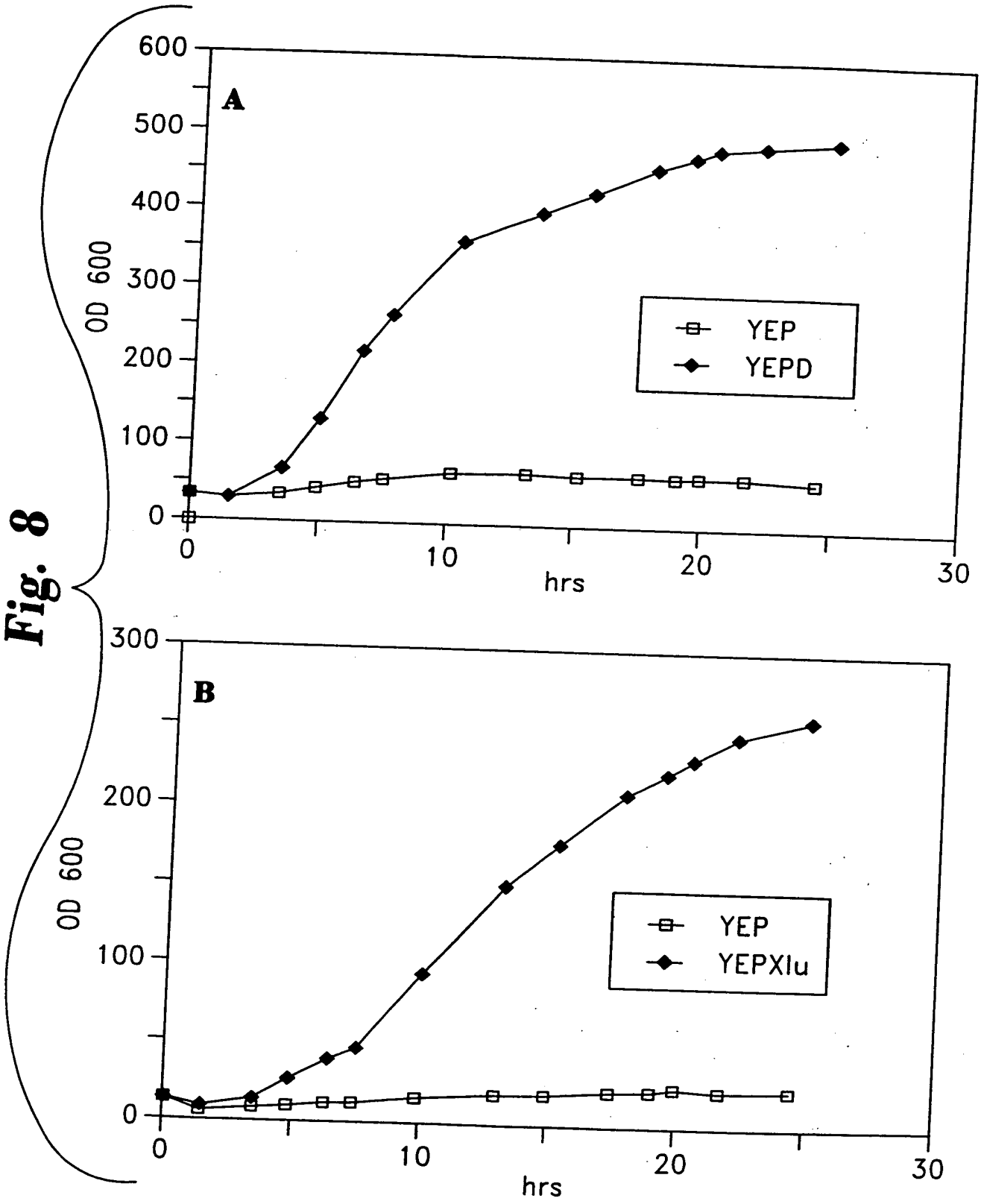
Fig. 6



Fermentation of glucose and xylose by LNH-ST(1) after being cultured for 4, 20, and 40 generations in non-selective (glucose) medium.

Fig 7

Fig. 8



(A) Yeast (S. cerevisiae) AH22 cultured in YEPD (1% yeast extracts, 2% peptone, 2% glucose or YEP (1% yeast extracts, 2% peptone).
(B) Yeast AH22 cultured in YEPXlu (1% yeast extracts, 2% peptone, 2% xylulose) or YEP.

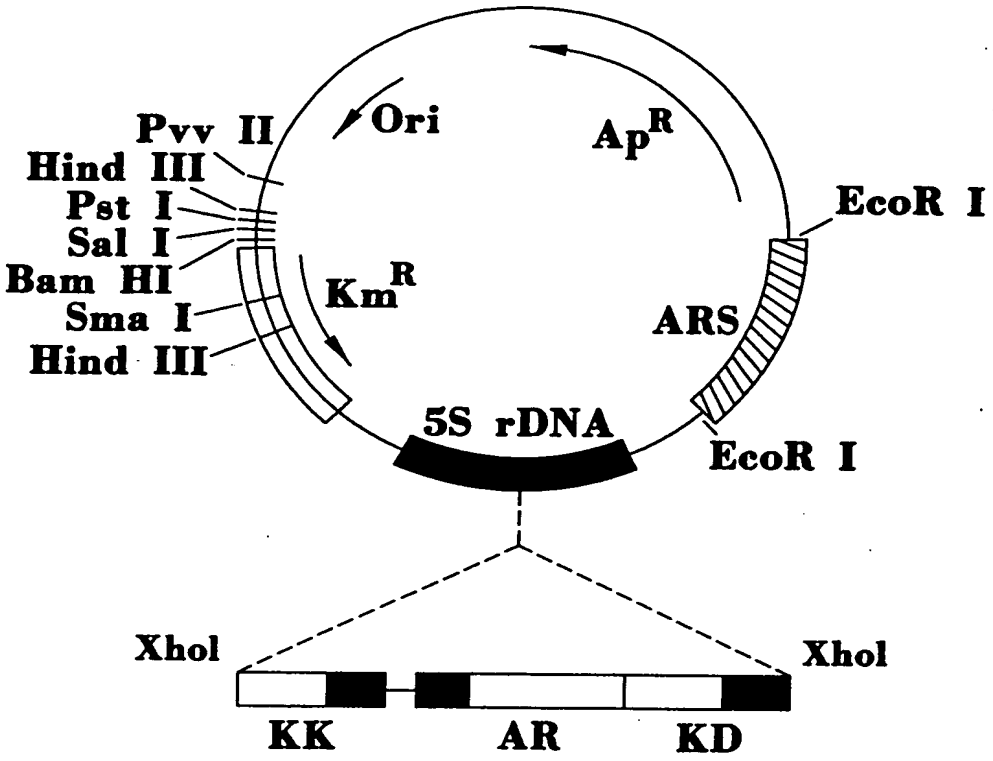

Pvv II
Hind III
Pst I
Sal I
Bam HI
Sma I
Hind III
Ori
Ap^R
EcoR I
Km^R
ARS
5S rDNA
EcoR I
XhoI
XhoI
KK
AR
KD

Fig. 9a

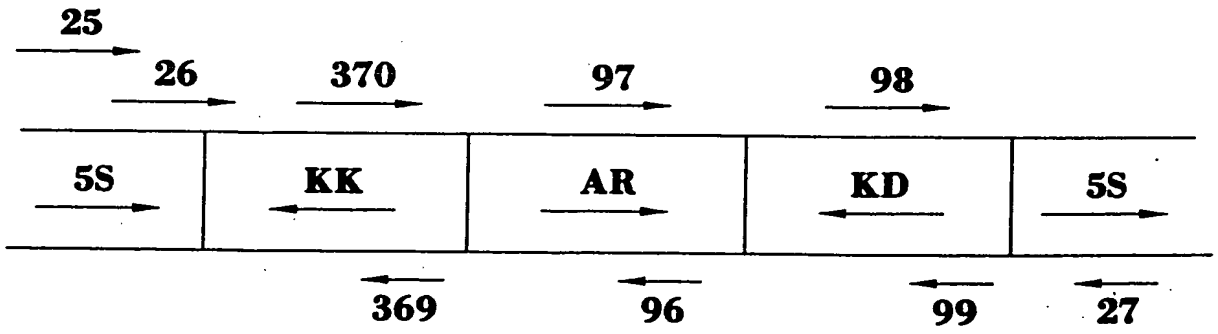

25
26
370
97
98
5S
KK
AR
KD
5S
369
96
99
27

Fig. 9b

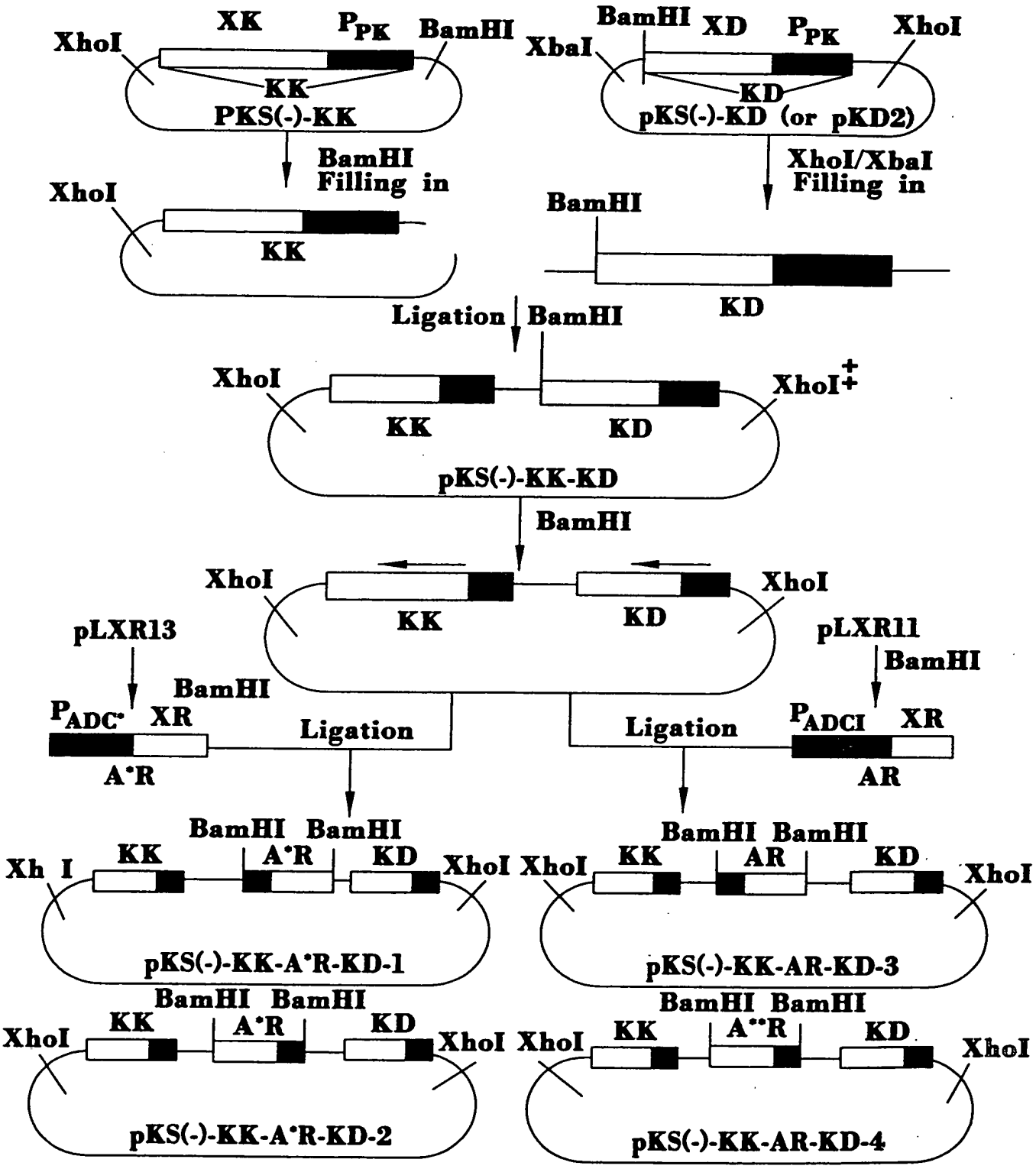
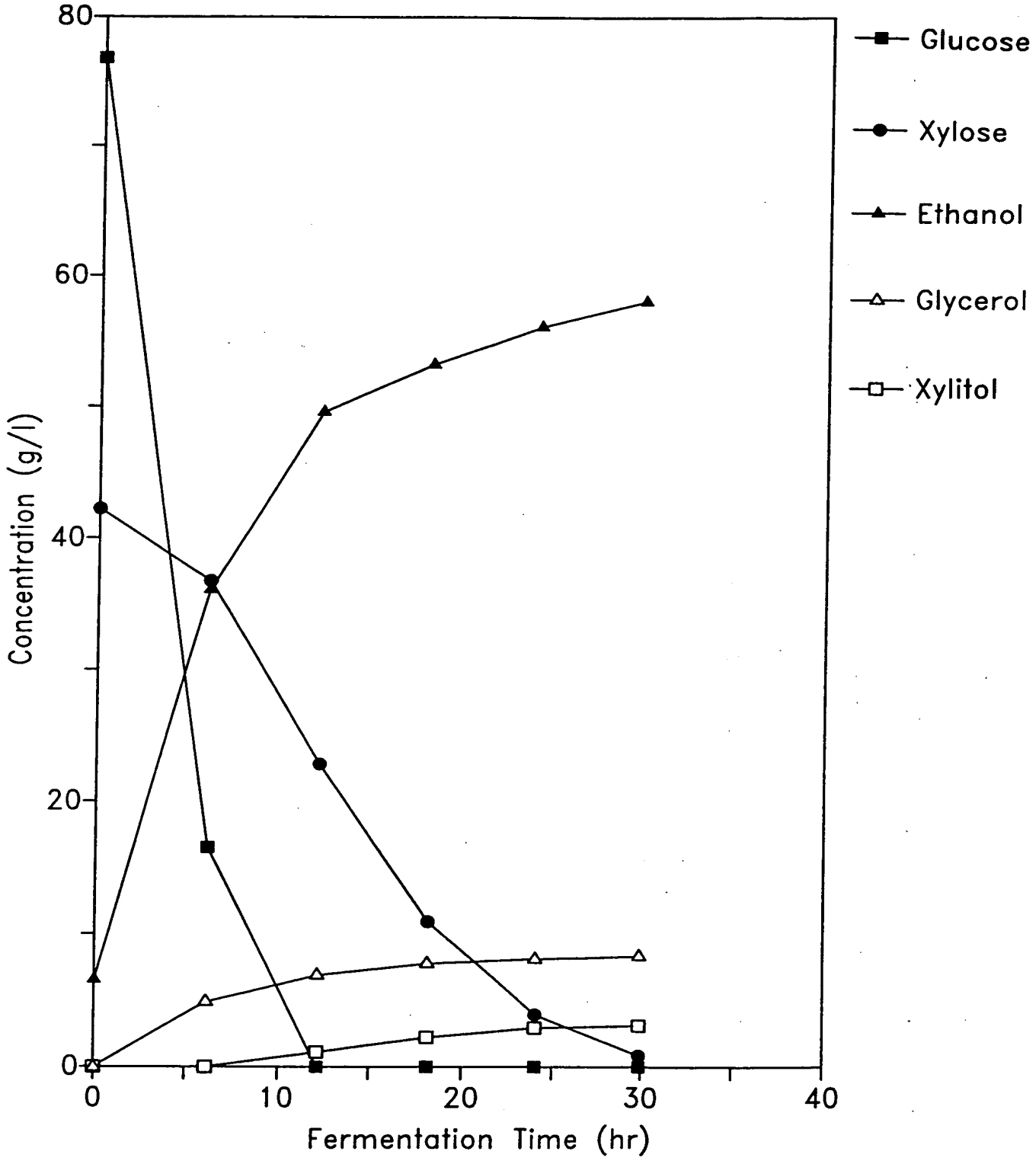


Fig. 10

Construction of pKS(−)−KK−AR−KD plasmids

+ The XhoI site was regenerated after ligation; *Intact ADC1 promoter;
** ADC1 promoter with TRP5 ribosomal binding site



Recombinant Saccharomyces 1400(LNH-ST)
for fermenting Glucose and Xylose

Fig. 11

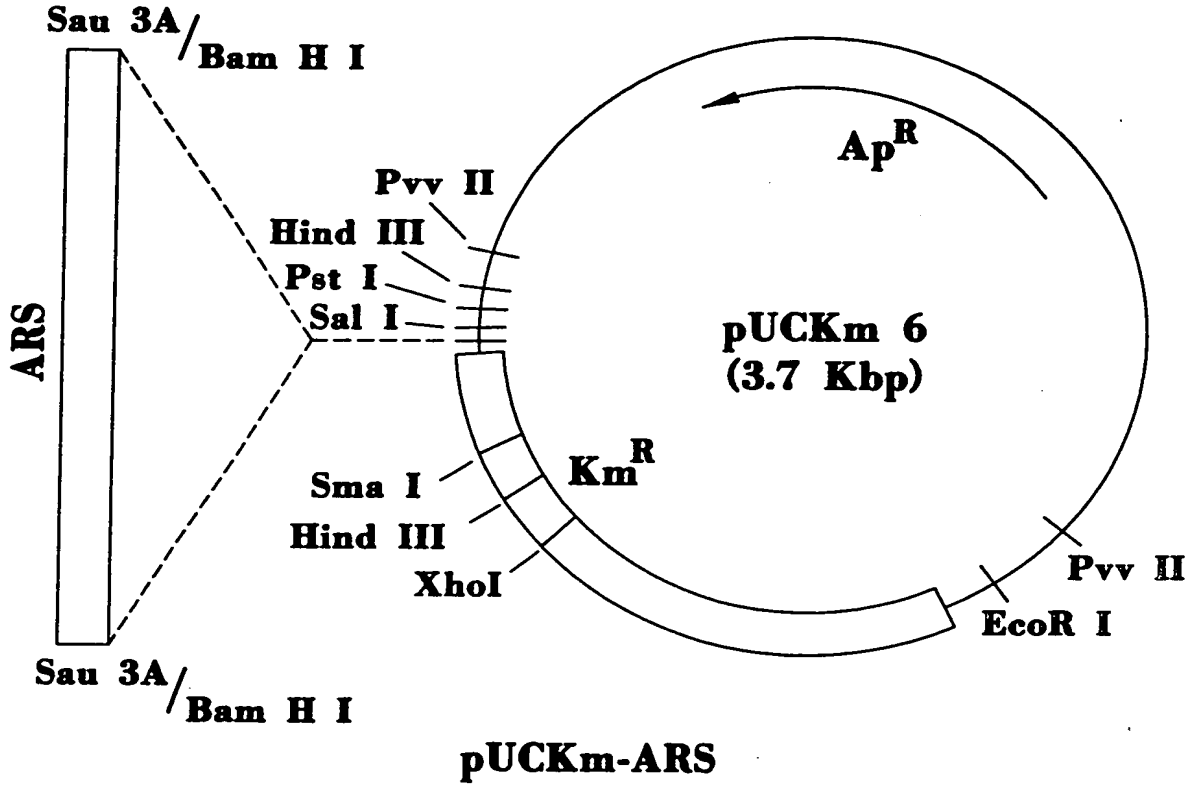

Sau 3A/Bam H I
ARS
Pvv II
Hind III
Pst I
Sal I
Sma I
Hind III
XhoI
Km^R
Ap^R
pUCKm 6
(3.7 Kbp)
Pvv II
EcoR I
Sau 3A/Bam H I
pUCKm-ARS

Fig. 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/07663

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, Dialog files 301, 155, 5, 351 (Chemname, Medline, Biosis, Derwent WPI)
search terms: Xyulokinase, xylose reductase, xylitol dehydrogenase, vector?, plasmid?, (ribosom? or 18Sor 28S(3n)(gene? ? or chromosom? or locus or loci or cistron), integrat?, insert?, yeast, Saccharomyces, Candida, Pichia, Pachysolen, ferment? degrad?, xylose, nonselect?

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 1/16, 1/18, 1/19, 15/09, 15/68, 15/69, 15/81, C12P 7/06

A1

(11) Internati nal Publication Number: **WO 97/42307**

(43) International Publication Date: 13 November 1997 (13.11.97)

(21) International Application Number: PCT/US97/07663

(22) International Filing Date: 6 May 1997 (06.05.97)

(30) Priority Data:
60/016,865 6 May 1996 (06.05.96) US

(71) Applicant *(for all designated States except US)*: PURDUE RESEARCH FOUNDATION [US/US]; Purdue University, Room 328, 1650 Engineering Administration Building, West Lafayette, IN 47907 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: HO, Nancy, W., Y. [US/US]; 606 Riley Lane, West Lafayette, IN 47906 (US). CHEN, Zheng-Dao [CN/US]; 480 Maple Street, West Lafayette, IN 47906 (US).

(74) Agents: GANDY, Kenneth, A. et al.; Woodard, Emhardt, Naughton, Moriarty & McNett, Bank One Center/Tower, Suite 3700, 111 Monument Circle, Indianapolis, IN 46204 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: STABLE RECOMBINANT YEASTS FOR FERMENTING XYLOSE TO ETHANOL

(57) Abstract

Described are recombinant yeast which ferment xylose to ethanol and which maintain their ability to do so when cultured for numerous generations in non-selective media. The preferred yeast contain multiple copies of integrated genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase fused to promoters which are non-glucose inhibited and which do not require xylose for induction. Also described are preferred methods for integrating multiple copies of exogenous DNA into host cells by transforming cells with replicative/integrative vectors, and then replicating the cells a number of times under selective pressure to promote retention of the vector in subsequent generations. The replicated vectors thus serve to integrate multiple copies of the exogenous DNA into the host cells throughout the replication/selection phase. Thereafter the selective pressure can be removed to promote loss of the vector in subsequent generations, leaving stable integrants of the exogenous DNA.

FOR THE PURPOSES OF INFORMATION ONLY

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PCT/US97/07663

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION OF PRIORITY DOCUMENTS

(PCT Administrative Instructions, Section 411)

To:
GANDY, Kenneth, A.
Woodard, Emhardt, Naughton,
Moriarty & McNett
Bank One Center/Tower
Suite 3700
111 Monument Circle
Indianapolis, IN 46204
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year)	
16 June 1997 (16.06.97)	
Applicant's or agent's file reference	**IMPORTANT NOTIFICATION**
PUR-48:PCT	

International application No.	International filing date (day/month/year)	Priority date (day/month/year)
PCT/US97/07663	06 May 1997 (06.05.97)	06 May 1996 (06.05.96)

Applicant
PURDUE RESEARCH FOUNDATION et al

Th applicant is hereby notified of the date of receipt by the International Bureau of the priority document(s) relating to th following application(s):

Priority application No:	Priority date:	Priority country:	Date of receipt of priority document:
60/016,865	06 May 1996 (06.05.96)	US	13 Jun 1997 (13.06.97)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimil No.: (41-22) 740.14.35

Auth rized fficer
Panakal

Telephon No.: (41-22) 338.83.38

Form PCT/IB/304 (July 1992)

001554780

PCT/US97/07663

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:
GANDY, Kenneth, A.
Woodard, Emhardt, Naughton,
Moriarty & McNett
Bank One Center/Tower
Suite 3700
111 Monument Circle
Indianapolis, IN 46204
ETATS-UNIS D'AMERIQUE

Dat of mailing (day/month/year)	
13 November 1997 (13.11.97)	
Applicant's or agent's file reference	**IMPORTANT NOTICE**
PUR-48:PCT	

International application No.	International filing date (day/month/year)	Priority date (day/month/year)
PCT/US97/07663	06 May 1997 (06.05.97)	06 May 1996 (06.05.96)

Applicant
PURDUE RESEARCH FOUNDATION et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,BR,CA,CN,EP,IL,JP,KP,KR,NO,PL,SK,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AL,AM,AP,AT,AZ,BA,BB,BG,BY,CH,CU,CZ,DE,DK,EA,EE,ES,FI,GB,GE,HU,IS,KE,KG,KZ,LC,LK,
   LR,LS,LT,LU,LV,MD,MG,MK,MN,MW,MX,NZ,OA,PT,RO,RU,SD,SE,SG,SI,TJ,TM,TR,TT,UA,UG,UZ,
   VN,YU
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   13 November 1997 (13.11.97) under No. WO 97/42307

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra
Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

From the INTERNATIONAL BUREAU

To:

GANDY, Kenneth, A.
Woodard, Emhardt, Naughton, Moriarty & McNett
Bank One Center/Tower
Suite 3700
111 Monument Circle
Indianapolis, IN 46204
ETATS-UNIS D'AMERIQUE

INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

Date of mailing (day/month/year)	
12 February 1998 (12.02.98)	
Applicant's or agent's file reference	**IMPORTANT INFORMATION**
PUR-48:PCT	

International application No.	International filing date (day/month/year)	Priority date (day/month/year)
PCT/US97/07663	06 May 1997 (06.05.97)	06 May 1996 (06.05.96)

Applicant
PURDUE RESEARCH FOUNDATION et al

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

   AP :GH,KE,LS,MW,SD,SZ,UG
   EP :AT,BE,CH,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
   National :AU,BG,BR,CA,CN,CZ,DE,FI,GB,IL,JP,KP,KR,MN,NO,NZ,PL,RO,RU,SE,SK, US,VN

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

   EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
   OA :BF,BJ,CF,CG,CI,CM,GA,GN,ML,MR,NE,SN,TD,TG
   National :AL,AM,AT,AZ,BA,BB,BY,CH,CU,DK,EE,ES,GE,HU,IS,KE,KG,KZ,LC,LK,LR, LS,LT,LU,LV,MD,MG,MK,MW,MX,PT,SD,SG,SI,TJ,TM,TR,TT,UA,UG,UZ,YU

3. The applicant is reminded that he must enter the "national phase" **before the expiration of 30 months from the priority date** before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing , if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

   Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

   The entry into the European regional phase is postponed **until 31 months from the priority date** for all States designated for the purposes of obtaining a European patent.

The International Bureau of WIPO
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1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer:

A. Addae-Ruesch

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

RECEIVED
JAN 30 1998
Woodard, Emhardt, Naughton, Moriarty & McNett

PCT

NOTIFICATION OF RECEIPT OF DEMAND

(PCT Rule 61.1(b), first sentence and Administrative Instructions, Section 601)

To:

KENNETH A. GANDY
WOODARD, EMHARDT, NAUGHTON, MORIARTY &
BANK ONE CENTER/TOWER, SUITE 3700
111 MONUMENT CIRCLE
INDIANAPOLIS IN 46204

Date of mailing *(day/month/year)*	26 JAN 1998
Applicant's or agent's file reference: PUR-48:PCT	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US97/07663	06 MAY 97	06 MAY 96

Applicant: PURDUE RESEARCH FOUNDATION

1. The applicant is hereby notified that this International Preliminary Examining Authority considers the following date as th date of receipt of the demand for international preliminary examination of the international application:

   05 DEC 1997

2. This date of receipt is:

   ☒ the actual date of receipt of the demand.

   ☐ the date on which the proper corrections to the demand were timely received.

3. ☐ This date is **AFTER** the expiration of 19 months from the priority date.

   Attention: The election(s) made in the demand does (do) not have the effect of postponing the commencement of the national phase until 30 months from the priority date (or later in some Offices) (Article 39(1)). Therefore, the acts for entry into the national phase must be performed within 20 months from the priority date (or later in some Offices) (Article 22).

   For details, see Annex B to Form PCT/IB/301 sent by the International Bureau and Volume II of the PCT Applicant's Guide.

   ☐ This notification confirms the information given in person or by telephone on:

   _____

4. Only where paragraph 3 applies, a copy of this notification has been sent to the International Bureau.

Name and mailing address of the IPEA/US	Authorized officer
Assistant Commissioner for Patents Box PCT Washington, D.C. 20231 Attn: IPEA/US Facsimile No.	PAUL F. URRUTIA Telephone No. 703·305·3681

Form PCT/IPEA/402 (July 1992)

PCT INTERNATIONAL APPLICATION TRANSMITTAL LETTER

DATE: 06 May 1997

REGARDING THE INTERNATIONAL APPLICATION OF
PURDUE RESEARCH FOUNDATION

DOCKET OR REFERENCE NUMBER
PUR-48:PCT

ENTITLED
STABLE RECOMBINANT YEASTS FOR FERMENTING XYLOSE TO ETHANOL

Certification under 37 CFR 1.10 (if applicable)

TB861763749US
"Express Mail" mailing number

06 May 1997
Date of Deposit

I hereby certify that this application is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231.

Leslie W. CURRY
(Typed or printed name of person mailing application)

Leslie W Curry
(Signature of person mailing application)

To the United States Receiving Office (RO/US):
Accompanying this transmittal letter is the above-identified International application, including a completed Request form (PCT/RO/101). Please process the application according to the provisions of the Patent Cooperation Treaty.

The following requests are made of the RO/US:

1. [X] PREPARATION AND TRANSMITTAL OF CERTIFIED COPY OF PRIORITY DOCUMENTS—Please prepare and transmit to the International Bureau a certified copy of the United States origin priority documents identified in Box VI of the Request form (37 CFR 1.451).

   To cover the cost of copy preparation and certification (37 CFR 1.19(a)(3) and (b)(1)),
   [X] a (check) (money order) in the amount of $ 15.00 included in fee is attached to this transmittal letter.
   [ ] the RO/US is hereby authorized to charge the following deposit account no.: _____.

2. [X] CHOICE OF INTERNATIONAL SEARCHING AUTHORITY—It is requested that the International Search be performed by the following International Searching Authority:
   [X] United States Patent and Trademark Office (ISA/US)
   [ ] European Patent Office (ISA/EP)

   The appropriate Search fee for the above-named Authority is indicated on the Fee Calculation Sheet (PCT/RO/101 Annex).

3. [X] SUPPLEMENTAL SEARCH FEES (ONLY WHEN ISA/US CONDUCTS THE INTERNATIONAL SEARCH.)—Please charge any Supplemental Search fees that may be required by the United States International Searching Authority (ISA/US) to deposit account no.: 23-3030.

   I understand that this authorization is subject to my oral confirmation thereof in each instance and that it in no way limits my right to submit a protest against payment of the Supplemental Search fees, but is merely an administrative aid to assure that the ISA/US may timely complete the Search Report

NOTE: SUPPLEMENTAL SEARCH FEES FOR ISA/EP ARE PAYABLE DIRECTLY TO THE EUROPEAN PATENT OFFICE

4. [X] DISCLOSURE INFORMATION—In order to assist in screening the accompanying International application for purposes of determining whether a license for foreign transmittal should and could be granted and for other purposes, the following information is supplied:
   A. [ ] There is no prior filed application relating to this invention.
   B. [X] There is a prior application, serial number 60/016,865 filed on 06 May 1996 (06.05.9
      which contains subject matter that is
      1. [X] substantially identical to that of the accompanying International application.
      2. [ ] less than that of the accompanying International application. The additional subject matter of the International application appears on pages(s) and line(s) _____.
      3. [ ] more than that of the accompanying International application.
   C. [ ] Disclosure information cannot be covered by the language of Points 4A or 4B above due to the involvement of several prior applications or for other reasons. A separate sheet on which the disclosure information is explained is attached to this transmittal letter.

5. [ ] REQUEST FOR FOREIGN TRANSMITTAL LICENSE—According to the provisions of 35 U.S.C. 184 and 37 CFR 5.11, a license to transmit the accompanying International application to foreign agencies or international authorities is hereby requested.

SIGNER IS THE
[ ] APPLICANT
[ ] COMMON REPRESENTATIVE
[X] (ATTORNEY) (AGENT) REG NO 33,386

NAME OF SIGNER (Typed)
Kenneth A. GANDY

SIGNATURE
Kenneth A Gandy

This sheet is part of and does not count as a sheet of the international application

PCT

FEE CALCULATION SHEET

Annex to the Request

For receiving Office use only

International application No.

Date stamp of the receiving Office

Applicant's or agent's file reference: PUR-48:PCT

Applicant: PURDUE RESEARCH FOUNDATION

CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE . . . . . 230 [T]

2. SEARCH FEE . . . . . 440 [S]

International search to be carried out by US

(If two or more International Searching Authorities are competent in relation to the international application, indicate the name of the Authority which is chosen to carry out the international search.)

3. INTERNATIONAL FEE

Basic Fee

The international application contains 68 sheets.

first 30 sheets . . . . . 590 [b_1]

38 (remaining sheets) x 12 (additional amount) = 456 [b_2]

Add amounts entered at b_1 and b_2 and enter total at B . . . . 1046 [B]

Designation Fees

The international application contains 69 designations.

69 (number of designation fees payable *(maximum 11)*) x 143 (amount of designation fee) = Max. 1573 [D]

Add amounts entered at B and D and enter total at I . . . . . 2619 [I]

(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and D.)

4. FEE FOR PRIORITY DOCUMENT . . . . . 15 [P]

5. TOTAL FEES PAYABLE

Add amounts entered at T, S, I and P, and enter total in the TOTAL box . . $ 3304.00 TOTAL

☐ The designation fees are not paid at this time.

MODE OF PAYMENT

[X]	authorization to charge deposit account (see below)	☐	bank draft	☐	coupons
[X]	cheque	☐	cash	☐	other *(specify)*:
☐	postal money order	☐	revenue stamps		

DEPOSIT ACCOUNT AUTHORIZATION *(this mode of payment may not be available at all receiving Offices)*

The RO/ US

☐ is hereby authorized to charge the total fees indicated above to my deposit account.

[X] is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

☐ is hereby authorized to charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account.

23-3030 — Deposit Account Number

6 May 1997 — Date *(day/month/year)*

Kenneth A. Gandy — Signature Kenneth A. GANDY 33,386

See Notes to the fee calculation sheet

Continuation of Box No. III   FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS

If none of the following sub-boxes is used, this sheet is not to be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

CHEN, Zheng-Dao
480 Maple Street
West Lafayette, Indiana 47906 US

This person is:
- [ ] applicant only
- [x] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: China

State *(i.e. country)* of residence: US

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [x] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality:

State *(i.e. country)* of residence:

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [ ] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality:

State *(i.e. country)* of residence:

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [ ] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality:

State *(i.e. country)* of residence:

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [ ] the United States of America only
- [ ] the States indicated in the Supplemental Box

- [ ] Further applicants and/or (further) inventors are indicated on another continuation sheet.

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* PUR-48:PCT

Box No. I TITLE OF INVENTION

STABLE RECOMBINANT YEASTS FOR FERMENTING XYLOSE TO ETHANOL

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

PURDUE RESEARCH FOUNDATION
1650 Engineering Administration Building, Room 328
Purdue University
West Lafayette, Indiana 47907
United States of America

☐ This person is also inventor.

Telephone No. 317-494-2610

Facsimile No.

Teleprinter No.

State *(i.e. country)* of nationality: US

State *(i.e. country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

HO, Nancy W.Y.
606 Riley Lane
West Lafayette, Indiana 47906
United States of America

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: US

State *(i.e. country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

GANDY, Kenneth A.
WOODARD, EMHARDT, NAUGHTON, MORIARTY & MCNETT
Bank One Center/Tower, Suite 3700
111 Monument Circle
Indianapolis, Indiana 46204 US

SEE CONTINUATION TO BOX NO. IV ON SHEET NO. 4

Telephone No. 317-634-3456

Facsimile No. 317-637-7561

Teleprinter No. 810-341-3283

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

- [X] AP **ARIPO Patent:** KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SZ Swaziland, UG Uganda, and any other State which is a Contracting State of the Harare Protocol and of the PCT Gh. Ghana
- [X] EA **Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- [X] EP **European Patent:** AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- [X] OA **OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- [X] AL Albania
- [X] AM Armenia
- [X] AT Austria
- [X] AU Australia
- [X] AZ Azerbaijan
- [X] BA Bosnia and Herzegovina
- [X] BB Barbados
- [X] BG Bulgaria
- [X] BR Brazil
- [X] BY Belarus
- [X] CA Canada
- [X] CH and LI Switzerland and Liechtenstein
- [X] CN China
- [X] CU Cuba
- [X] CZ Czech Republic
- [X] DE Germany
- [X] DK Denmark
- [X] EE Estonia
- [X] ES Spain
- [X] FI Finland
- [X] GB United Kingdom
- [X] GE Georgia
- [X] HU Hungary
- [X] IL Israel
- [X] IS Iceland
- [X] JP Japan
- [X] KE Kenya
- [X] KG Kyrgyzstan
- [X] KP Democratic People's Republic of Korea
- [X] KR Republic of Korea
- [X] KZ Kazakstan
- [X] LC Saint Lucia
- [X] LK Sri Lanka
- [X] LR Liberia
- [X] LS Lesotho
- [X] LT Lithuania
- [X] LU Luxembourg
- [X] LV Latvia
- [X] MD Republic of Moldova
- [X] MG Madagascar
- [X] MK The former Yugoslav Republic of Macedonia
- [X] MN Mongolia
- [X] MW Malawi
- [X] MX Mexico
- [X] NO Norway
- [X] NZ New Zealand
- [X] PL Poland
- [X] PT Portugal
- [X] RO Romania
- [X] RU Russian Federation
- [X] SD Sudan
- [X] SE Sweden
- [X] SG Singapore
- [X] SI Slovenia
- [X] SK Slovakia
- [X] TJ Tajikistan
- [X] TM Turkmenistan
- [X] TR Turkey
- [X] TT Trinidad and Tobago
- [X] UA Ukraine
- [X] UG Uganda
- [X] US United States of America
- [X] UZ Uzbekistan
- [X] VN Viet Nam

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

- [X] YU Yugoslavia
- [ ] .....
- [ ] .....
- [ ] .....

In addition to the designations made above, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except the designation(s) of _____.

The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Supplemental Box *If the Supplemental Box is not used, this sheet need not be included in the request.*

Use this box in the following cases:

1. If, in any of the Boxes, the space is insufficient to furnish all the information:	***in such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient;***
in particular:	
(i) if more than two persons are involved as applicants and/or inventors and no "continuation sheet" is available:	*in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III;*
(ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication "the States indicated in the Supplemental Box" is checked:	*in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;*
(iii) if, in Box No. II or in any of the sub-boxes of Box No. III, the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America:	*in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;*
(iv) if, in addition to the agent(s) indicated in Box No. IV, there are further agents:	*in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;*
(v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication "patent of addition," or "certificate of addition," or if, in Box No. V, the name of the United States of America is accompanied by an indication "Continuation" or "Continuation-in-part":	*in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;*
(vi) if there are more than three earlier applications whose priority is claimed:	*in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI.*
2. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning non-prejudicial disclosures or exceptions to lack of novelty:	***in such case, write "Statement Concerning Non-Prejudicial Disclosures or Exceptions to Lack of Novelty" and furnish that statement below.***

Continuation to Box No. IV Agent

WOODARD, Harold R.; EMHARDT, C. David; NAUGHTON, Joseph A., Jr.; MORIARTY, John V.; McNETT, John C.; HENRY, Thomas Q.; DURLACHER, James M.; REEVES, Charles R.; WAGNER, Vincent O.; ZLATOS, Steve; BEREVESKOS, Spiro; BAHRET, William F.; BROWNING, Clifford W.; FRISK, R. Randall; LUEDERS, Daniel J.; BECK, Michael D.; GANDY, Kenneth A.; THOMAS, Timothy N.; SISSELMAN, Kerry P.; JONES, Kurt N.; ALLIE, John H.; MICHAEL, Jeffrey A.; BECK, Deborah R.; BANTA, Holiday W.; COLE, Troy J.; PAYNTER, L. Scott; HIDAY, Lisa A. and ROWE, James L., all of Woodard, Emhardt, Naughton, Moriarty & McNett, Bank One Center/Tower, Suite 3700, 111 Monument Circle, Indianapolis, Indiana 46204 United States of America

Box No. VI PRIORITY CLAIM Further priority claims are indicated in the Supplemental Box ☐

The priority of the following earlier application(s) is hereby claimed:

Country *(in which, or for which, the application was filed)*	Filing Date *(day/month/year)*	Application No.	Office of filing *(only for regional or international application)*
item (1) US	06 May 1996 (06.05.96)	60/016,865	
item (2)			
item (3)			

Mark the following check-box if the certified copy of the earlier application is to be issued by the Office which for the purposes of the present international application is the receiving Office (a fee may be required):

☒ The receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s) : (1)

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(If two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):* ISA / US

Earlier search *Fill in where a search (international, international-type or other) by the International Searching Authority has already been carried out or requested and the Authority is now requested to base the international search, to the extent possible, on the results of that earlier search. Identify such search or request either by reference to the relevant application (or the translation thereof) or by reference to the search request:*

Country (or regional Office):	Date *(day/month/year)*:	Number:
US	06 May 1996 (06.05.96)	60/016,865

Box No. VIII CHECK LIST

This international application contains the following number of sheets:

1. request	: 5	sheets
2. description	: 44	sheets
3. claims	: 6	sheets
4. abstract	: 1	sheets
5. drawings	: 12	sheets
Total	: 68	sheets

This international application is accompanied by the item(s) marked below:

1. ☐ separate signed power of attorney
2. ☐ copy of general power of attorney
3. ☐ statement explaining lack of signature
4. ☐ priority document(s) *identified in Box No. VI as item(s):*
5. ☒ fee calculation sheet
6. ☐ separate indications concerning deposited microorganisms
7. ☐ nucleotide and/or amino acid sequence listing (diskette)
8. ☒ other *(specify)*: Transmittal Letter (dup)

Figure No. 6 of the drawings (if any) should accompany the abstract when it is published.

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Applicant:
HO, Nancy W.Y.
CHEN, Zheng-Dao

PURDUE RESEARCH FOUNDATION

Agent: Kenneth A. Gandy

(Kenneth A. GANDY)

For receiving Office use only

1. Date of actual receipt of the purported international application:	2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:	
4. Date of timely receipt of the required corrections under PCT Article 11(2):	
5. International Searching Authority specified by the applicant: ISA /	6. ☐ Transmittal of search copy delayed until search fee is paid

For International Bureau use only

Date of receipt of the record copy by the International Bureau: